Humoral immune response to melanoma: discovery and evaluation of anti-melanoma antibodies

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Abstract

Melanoma, a potentially lethal form of skin cancer, is widely thought to be immunogenic in nature. While numerous studies have examined T cell-mediated immune responses to melanoma and their therapeutic potential, there has been less focus on B cell-mediated immune responses and the tumor-reactive antibodies they produce. The aim of this work was three-fold: (1) to develop a methodology to detect antibodies secreted by human B cells that recognize melanoma cell surface proteins; (2) to evaluate the mature B cell repertoire of individuals with melanoma for antibody subclass composition and the presence and prevalence of anti-tumor antibodies; and (3) to study patient-derived antibodies and two engineered antibodies recognizing melanoma cells for their propensity to activate immune effector cells and their capacity to kill or restrict the growth of tumor cells.

As part of this thesis, a novel tumor cell-based ELISA was developed for the detection of tumor-reactive antibodies. Utilizing this new assay, the presence and prevalence of melanoma-reactive IgG antibodies derived from *ex vivo* cultured peripheral blood B cells from a cohort of 21 patients with melanoma (Stage I, n=1; Stage II, n=8; Stage III, n=6; Stage IV, n=6) were compared to those from healthy volunteers (n=10). While B cells from melanoma patients secreted IgG antibody concentrations comparable to those from healthy volunteer B cell cultures, a significantly increased reactivity of antibodies derived from patients to primary and metastatic melanoma cells was measured compared to healthy volunteers ($P<0.001$). Interestingly, there was a significant reduction in antibody responses to melanoma with advancing disease stage that was not found to be solely due to a

reduction in the B cell memory compartment size. Comparing IgG antibody subclass distribution among cutaneous tumors, patient lymph nodes and peripheral blood B cells all isolated from individuals with metastatic disease, elevated proportions of IgG4 subclass antibodies were observed in cutaneous tumors which present a novel finding of this thesis. These findings point to differentially polarized humoral immune responses in cutaneous tumor microenvironments.

Lastly, an antibody derived from a patient was then selected and preliminary evaluations of reactivity and specificity to a range of melanoma cell lines and primary human melanocytes were conducted. Using a live cell imaging cytotoxicity assay, this patient-derived melanoma-specific antibody was observed to kill melanoma cells via antibody-mediated cellular cytotoxicity. Additionally, two engineered monoclonal antibodies recognizing a melanoma associated antigen were found to partially restrict tumor cell migration and adhesion and to kill melanoma cells via antibody-dependent cellular cytotoxicity or phagocytosis.

In summary, examining the humoral immune response to melanoma and the effector function of antibodies targeting melanoma cells provides insight into the discovery of new therapeutic strategies for the treatment of melanoma.

Table of Contents

Table of Figures

List of Tables

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
ANOVA	Analysis of variance
APC	Allophycoyanin
BCR	B cell receptor
Bregs	Regulatory B cells
CD	Cluster of differentiation
CDC	Complement dependent cytotoxicity
cDNA	Complementary deoxyribonucleic acid
CDR	Complementarily determining region
CFSE	Carboxyfluorescein diacetate,succinimidyl ester
C_H	Heavy chain constant domain
C_L	Light chain constant domain
CI	Confidence interval
CSPG4	Chondroitin sulfate proteoglycan 4
CTLA-4	Cytotoxic lymphocyte antigen 4
DAB	3,3' Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DPX	Neutral mounting medium
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic
EGFR	Human epidermal growth factor receptor
ELISA	Enzyme-linked immunoabsorbant assay
ERK	Extracellular signal-regulated kinase
Fab	Fragment antigen binding
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
Fc	Fragment crystallizable
FcR	Fc receptor
FcRn	Neonatal Fc receptor
FCS	Fetal calf serum
FcαR	Fc alpha receptor
FcγR	Fc gamma receptor
FcεR	Fc epsilon receptor
FDA	Food and Drug Administration
FITC	Fluorescein isothiocynanate
FRα	Folate receptor alpha

gp	Glycoprotein
HBSS	Hank’s Buffered Salt Solution
HCl	Hydrochloric acid
HER	Human epidermal growth factor receptor
Her2/neu	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HMW-MAA	High molecular weight melanoma associated antigen
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
ITIM	Tyrosine-based inhibitor motif
KCL	King's College London
LDA	Limiting dilution assay
mAb	Monoclonal antibody
MAGE	Melanoma associated antigen
MAPK	Mitogen-activated protein kinase
MCSP	Melanoma chondroitin sulfate proteoglycan
MEM	Minimum Essential Medium

MMP	Matrix metalloproteinase
NGS	Normal goat sera
NK	Natural killer
NY-ESO-1	New York esophageal squamous cell carcinoma 1
ODN	Oligodeoxynucleotides
OPD	σ-Phenylenediamine dihydrochloride
PBBL	Peripheral blood B lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with 0.05% Tween 20®
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PenStrep	Penicillin and Streptomycin
PE	Phycoerthrin
RNA	Ribonucleic acid
scFv	Single-chain fragments of the variable region
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus

SSX2	Synovial sarcoma
TAA	Tumor associated antigen
TBS	Tris buffered saline
Th1	T helper cell subset 1
Th2	T helper cell subset 2
TIL	Tumor-infiltrating lymphocytes
TIL-B	Tumor-infiltrating B lymphocytes
TLR	Toll-like receptor
Treg	Regulatory T cells
TYRP	Tyrosinase-related protein
VEGF	Vascular endothelial growth factor
V_H	Heavy chain variable domain
V_L	Light chain variable domain

Chapter 1: Introduction

1.1 Antibodies

The study of antibodies commenced in the 1890s with the seminal work of Emil von Behring and Shibasaburo Kitasato. Behring and Kitasato discovered that a non-cellular component of the blood of animals immune to tetanus toxoid and diphtheria could mount short anti-toxic activity in unimmunized individuals. This was the first evidence of an immune mediator in serum that could neutralize infections. Building upon this work, Paul Ehrlich in the beginning of the 20th century theorized that cells "grew additional side chains" (antibodies) that could bind to specific toxins and these side chains could break off and circulate through the body. Ehrlich postulated that these "side chains" could specifically seek out targeted cells without harming normal cells and postulated they were "magic bullets" to cure diseases. The scientific community to the present date has focused on elucidating the source, structure, function, and therapeutic utility of antibodies. The emergence of antibodies as an important class of drugs since the 1990s has shown some realization of Ehrlich's notion of antibodies as "magic bullets" for the treatment of many diseases, including cancer.

1.1.1 The Role of Antibodies in the Adaptive Immune Response

The immune system can mount a rapid response to foreign pathogens by the engagement of the innate immune system comprised of soluble factors such as complement proteins and cellular components including natural killer (NK) cells, macrophages/monocytes, eosinophils, basophils, neutrophils, mast cells and dendritic cells. These components of the innate immune system can recognize pathogens by pattern-recognition receptors and other surface molecules to

mediate the destruction of invading pathogens by proteolysis, phagocytosis, and the secretion of cytokines and/or chemokines leading to inflammation and the recruitment of other phagocytic cells such as neutrophils. While slower to develop, the adaptive arm of the immune system can also mount an immune response to pathogens but with increased antigenic specificity and memory compared to innate immune responses, taking effect within 4 to 7 days (Janeway, Travers et al. 2005). The adaptive immune response is mediated by two major types of lymphocytes: B and T cells. B cells can regulate adaptive immune responses in multiple ways such as by the secretion of meditators that drive T cell responses or by the production of antibodies specific for foreign antigens which could mount a humoral immune response (Lund and Randall 2010). These antibodies, once bound to a pathogen, can result in pathogen neutralization or can interact with cellular and non-cellular components of the innate immune system resulting in pathogen destruction. T cells specific for foreign antigens are capable of cell-mediated immunity either by direct killing via the perforin/granzyme pathways and death ligands of cells displaying foreign antigens (cluster of differentiation [CD]8+ T cells), or through the enhancement of antibody production, CD8+ T cell, NK cell or macrophage cytotoxic function (CD4+ T cells) against pathogen coated or infected cells.

Humoral immune responses, a primary focus of this thesis, are mediated by antibodies. Antibodies or immunoglobulin (Ig) are proteins produced by B cells in response to the presence of pathogens or foreign antigens. Antibodies can be membrane bound on the surface of B cells as an antigen receptor (B cell receptor or BCR) or secreted as soluble proteins, with each B cell producing antibodies with specificity for a single epitope of an antigen. B cells mature in the bone marrow,

and then continually circulate from the blood into peripheral lymphoid tissues such as the spleen, lymph nodes and mucosal lymphoid tissues. Once activated by an antigen in either a T cell dependent or independent manner, B cells can then undergo further expansion and clonal differentiation for the selection of high affinity antibodies following further rounds of mutation (somatic hypermutation) (Rajewsky 1996; Janeway, Travers et al. 2005). The activation of B cells to produce antibodies mostly occurs in a T cell dependent manner where CD4+ activated helper T cells can activate B cells that have encountered the same antigen. This activation of B cells by CD4+ T cells occurs through the interaction of C40 ligand on the surface of activated T cells and the CD40 protein on the B cell surface. In response to co-stimulatory molecules such as engagement of the CD40 receptor on B cells and by CD40 ligand (CD154) and the secretion of various cytokines, B cells will maintain antigen specificity but undergo class switching from IgM and IgD to IgA, IgG or IgE resulting in structural changes to the region of the molecule that interact with different immune effector molecules creating a tailored immune response.

Following activation by antigen in germinal centers, B cells can develop into memory B cells or plasma cells, regulated by antigen-dependent triggering of BCR, cytokines and direct interactions between T and B cells by molecules such as CD40 and CD27 (Agematsu, Nagumo et al. 1998). Some cells will become terminally differentiated plasma cells capable of secreting high levels of antibodies in an antigen-independent manner and other cells will become long lived memory B cells (Slifka, Antia et al. 1998). The conventional lineage of different B cell populations in the human body can be distinguished by the expression of different cell surface molecules such as CD molecules (Table 1.1), with CD27 being a widely

used marker to identify memory B cells (Klein, Rajewsky et al. 1998; Sato, Tuscano et al. 1998; Edwards and Cambridge 2006; Sanz, Wei et al. 2008; Manjarrez-Orduno, Quach et al. 2009). Memory B cells, generally producing antibodies with high affinity to antigen, can quickly mount a secondary immune response maintaining immunological memory following activation by antigen or antigen-independent polyclonal stimuli such as CpG deoxyribonucleic acid (DNA) to proliferate as antibody secreting cells (Bernasconi, Traggiai et al. 2002).

Table 1.1 Cell Surface Molecules Used to Identify Selected Conventional B cell Subpopulations in Humans

	Cell surface molecule	IgD	IgM	IgG/A/E	CD19	CD20	CD22	CD27	CD38	CD138
Bone marrow	Pro-B cell	-	-	-	+	-	-	-	-	-
	Pre-B cell	-	-	-	+	+	-	-	-	-
	Immature B cell	-	++	-	+	+	-	-	-	-
Peripheral compartments	Naïve	++	+	-	+	+	+	-	+/-	-
	Unswitched memory B cell	+	+	-	+	+	+	+	Low	-
	Switched memory B cell	-	-	+	+	+	+	+	Low	-
	Plasma blast	-	+/-	+	+	-	-	++	High	High
	Plasma cell†	-	-	+	+	-	-	++	+++	++

† Plasma cells can be found in both the bone marrow and peripheral compartments.
Modified from Sato, Tuscano et al. 1998; Janeaway, Travers et al., 2005; Edwards and Cambridge 2006; Manjarrez-Orduno, Quach et al. 2009.

There have been five major antibody classes or isotypes described in humans: IgM, IgD, IgG, IgA, and IgE, with each antibody class having distinct distribution in the body and roles in mediating immune responses. The first antibody class produced in a humoral immune response is IgM. IgM can be produced from B cells prior to class switching and tends to have low affinity to antigen compared to the class switched antibody classes, IgG/A/E. IgM can be expressed as a monomer on the surface of B cells or as a pentameter in soluble form with poor tissue diffusion due to its large size. IgM is found in the blood and lymphatic system and its effector functions are thought to primarily lie in the activation of the complement pathway. IgD is the second lowest antibody class expressed in the blood, >1% compared to the other antibody classes, and its function is the least understood. IgD is expressed on the surface of B cells as they exit the bone marrow and enter peripheral lymphoid tissues. Recent insight into the function of IgD has suggested that it may have pro-inflammatory and antimicrobial functions upon crosslinking on basophils (Chen, Xu et al. 2009). IgG is the most abundant antibody class in the body, it is secreted as a monomer primarily in the blood, and its primary function is to protect against bacterial and viral infections predominantly through the activation of complement pathways and opsonization of pathogens for engulfment by phagocytes. There have been four subclasses described for IgG: IgG1, IgG2, IgG3 and IgG4. IgA is found in mucosal membranes and secretions and also in the blood and it is thought to play a key role in mucosal immunity primarily through the neutralization of pathogens. IgA can be found as a monomer or in dimers and two subclasses have been described for this antibody class: IgA1 and IgA2. IgE is the least abundant antibody in the blood and it is found primarily in lungs, skin and mucous membranes as a monomer. IgE is involved in allergic reactions and immune responses to parasitic infections. IgE has high affinity to receptors on

immune cells, such as mast cells, and upon antigen binding, it can trigger these immune cells to release inflammatory mediators to induce allergic inflammation.

Antibodies have multiple mechanisms to engage an immune response against invading pathogens and foreign molecules. These mechanisms include the neutralization of pathogens preventing entrance into cells and replication, opsonization of extracellular bacteria, and activation of the complement system. Antibodies play a crucial role in directing phagocytic cells to pathogens and other foreign substances resulting in their ingestion, degradation and ultimate removal from the body. The aggregation or multimerization of antibodies in the presence of multivalent antigens can induce the crosslinking of antibody receptors on immune cells, resulting in high avidity binding and immune cell activation, whereas monomeric immunoglobulin engaged with monomeric antigens, or in the absence of antigen, has low affinity to antibody receptors on immune cells and cannot crosslink and activate immune cells. Antibodies in the presence of antigen play an important role in the adaptive immune responses because they are able to specifically target the destruction of foreign pathogens through the engagement of innate immune effector cells and complement proteins.

Humans can have a large repertoire of antibodies, with as many as 10^{11} antibodies per person (Janeway, Travers et al. 2005). Central to the maintenance of this large antibody repertoire is the role of circulating memory B cells able to mount a secondary antibody response to pathogens (Lanzavecchia, Bernasconi et al. 2006). The persistence of this cell pool, affinity to antigens, and the diversity of antigen specificity and effector mechanisms of antibodies collectively play a vital role in mounting an effective immune response against pathogens.

1.1.2 Antibody Structure and Fc Effector Function

Antibodies are large molecules: as monomers they range from 150 to 190 kDa in size. An antibody is composed of two identical heavy chains and two identical light chains (Figure 1.1). Antibody structure is relatively similar among the five Ig classes; however, they differ in three ways: (1) number of heavy chain domains, (2) distribution and number of carbohydrate groups, and (3) the number and location of disulfide bonds linking different domains. Heavy chains are comprised of one variable domain (V_H) and multiple constant domains (C_H), three for IgA, IgD, and IgG (C_H1, C_H2 and C_H3) and four for IgE and IgM (C_H1, C_H2, C_H3, and C_H4). Light chains are also composed of one variable domain (V_L) but in contrast to the heavy chain only have a single constant domain (C_L) for all antibody classes.

Antibodies have two distinct binding regions: the fragment antigen binding (Fab) region and the fragment crystallizable (Fc) region (Figure 1.1). The Fab and Fc binding regions of antibodies each have a separate functional role. Antigen binding sites are located in the variable domains of the Fab region. Each variable domain (V_H and V_L) contains three polypeptide loops termed complementarily determining regions (CDRs) which are the key determinants of the specificity and affinity of antigen binding. These CDRs are supported by four polypeptides in each V_H and V_L called framework regions, which form a structural scaffold for CDR presentation. While variable regions in the Fab region contain binding sites to antigens, the constant domains in the Fc regions primarily determine the effector functions of antibodies through their interaction with soluble proteins and Fc receptors (FcR) expressed on immune cells.

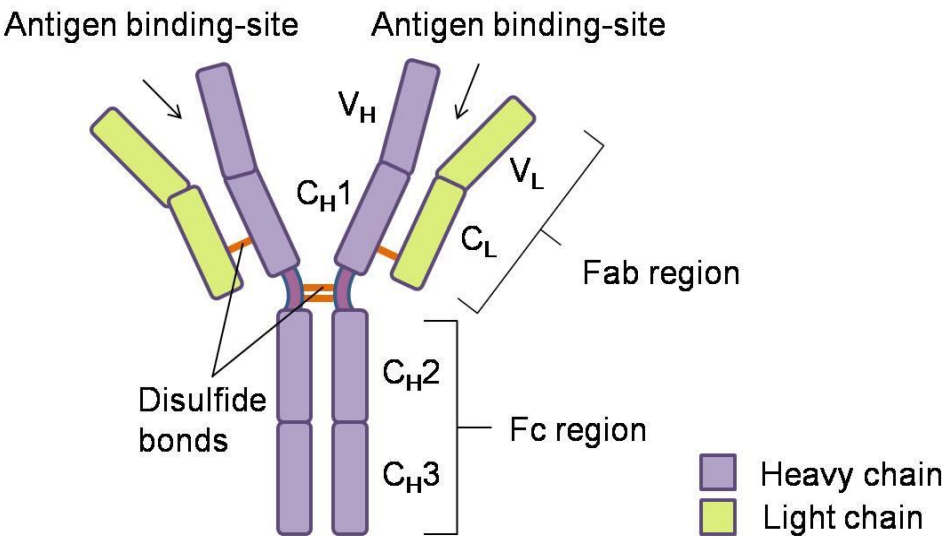


Figure 1.1 IgG structure. IgG molecules are composed of identical heavy and light protein chains which are linked by disulfide bonds (orange lines). Heavy chains consist of three constant domains (C_H1, C_H2, and C_H3) and one variable domain (V_H). Light chains also contain one variable domain (V_L) but only one constant domain (C_L). Antigen binding sites are located in the V_H and V_L domains of the Fab region. Effector function is mediated by the binding of the heavy chains in the Fc region, C_H2 and C_H3, to various components of the immune system**.**

The Fc structure of IgG and its affinity for different FcRs can confer different biological activities and regulate antibody distribution. For example, the concentration of IgG in circulation is regulated by the neonatal Fc receptor (FcRn) (Roopenian and Akilesh 2007). Fc regions of antibodies of the IgG class can also mediate immune effector functions by opsonization, complement activation, and the activation of immune cells following Fab binding. The ability of IgG to mount a cellular immune response is regulated through Fc receptors specific for the IgG Fc fragment (FcγR) expressed on immune cells such as monocytes/macrophages, neutrophils, dendritic cells, and NK cells (Janeway, Travers et al. 2005;

Nimmerjahn and Ravetch 2008). For IgG, there are three activating receptors, FcγRI (CD64), FcγRIIA (CD32) and FcγRIII (CD16); and one inhibitory receptor FcγRIIB (CD32) (Nimmerjahn and Ravetch 2008), with each receptor having differential expression on immune effector cells (Table 1.2). Upon aggregation by multivalent antibody-antigen complexes FcγRs present on immune cells can modulate biological activity. Each of the four IgG subclasses has a different Fc structure resulting in differential specificity and affinities for these FcγRs on immune cells (Table 1.2), contributing to the different biological activities attributed to each subclass (Bruhns, Iannascoli et al. 2009).

IgE is known to be recognized by two Fc epsilon receptors (FcεRs) expressed on immune cells: the low affinity receptor (CD23 or FcεRII) and the high affinity receptor (FcεRI) (Gould, Sutton et al. 2003). CD23 is expressed on hematopoietic cells such as B cells, macrophages, monocytes, and eosinophils. The higher affinity IgE Fc receptor, FcεRI, is expressed on mast cells and basophils as tetramers and on antigen-presenting cells such as dendritic cells and monocytes/macrophages as trimers in much lower amounts (Kraft and Kinet 2007). The affinity of IgE to FcεRI is 10^{10} M^{-1}, at least a hundred fold higher than the affinity of IgG for any FcγR or IgA for Fc receptor alpha I (FcαRI) (Table 1.2), and this high affinity of IgE to this receptor may result in the engagement of immune cells such as mast cells and basophils capable of producing a potent immune response including the release of inflammatory mediators and cytokines (Gould and Sutton 2008).

Table 1.2 Selected Properties of Fc Receptors on Innate Immune Cells Relevant for the Function of Therapeutic Monoclonal Antibodies

Fc receptor	Ligand	Affinity (Ka)*	Expression on key immune effector cells	Cellular effector mechanism upon ligation
FcαRI (CD89)	IgA	10^7 M^{-1}	Monocytes	ADCP
			Macrophages	ADCP
			Neutrophils	ADCC
			Eosinophils	ADCC
FcεRI	IgE	10^8-10^{11} M^{-1}	Mast cells	Degranulation/release of inflammatory mediators
			Basophils	
			Eosinophils	ADCC
			Monocytes	ADCC
FcεRII (CD23)	IgE	10^7-10^8 M^{-1}	Monocytes	ADCP
			B cells	Endocytosis, antigen presentation, ADCP
FcγRI (CD64)	IgG	IgG1=6.5 x 10^7 M^{-1}	Macrophages	ADCP
		IgG2=No binding	Monocytes	ADCP
		IgG3=6.1 x 10^7 M^{-1}	Neutrophils	ADCC
		IgG4=3.4 x 107 M^{-1}	Eosinophils	ADCC
			Dendritic cells	Phagocytosis and antigen presentation/T cell activation
FcγRIIA (CD32)	IgG	†IgG1=5.2 x 10^6 M^{-1}	Macrophages	ADCP
		IgG2=4.5 x 10^5 M^{-1}	Monocytes	ADCP
		IgG3=9.1 x 10^5 M^{-1}	Neutrophils	ADCP
		IgG4=2.1 x 10^5 M^{-1}	Eosinophils	ADCC
		†IgG1=3.5 x 10^6 M^{-1}		
		IgG2=1.0 x 10^5 M^{-1}		
		IgG3=1.7 x 10^5 M^{-1}		
		IgG4=2.0 x 10^5 M^{-1}		
FcγRIIB (CD32)	IgG	IgG1=1.2 x 10^5 M^{-1}	Macrophages	Inhibition of stimulation
		IgG2=0.2 x 10^5 M^{-1}	Mast Cells	
		IgG3=1.7 x 10^5 M^{-1}	Neutrophils	
		IgG4=2.0 x 10^5 M^{-1}	Eosinophils	Regulation of humoral immune response
			B cells	
FcγRIIIA (CD16)	IgG	††IgG1=1.2 x 10^6 M^{-1}	NK cells	ADCC
		IgG2=0.3 x 10^5 M^{-1}	Eosinophils	ADCC
		IgG3=7.7 x 10^6 M^{-1}	Monocytes	ADCP
		IgG4=2.0 x 10^5 M^{-1}	Macrophages	ADCP
		††IgG1=2.0 x 10^6 M^{-1}	Neutrophils	ADCP
		IgG2=0.7 x 10^5 M^{-1}	Mast cells	Degranulation/release of inflammatory mediators
		IgG3=9.8 x 10^6 M^{-1}		
		IgG4=2.5 x 10^5 M^{-1}		

*Affinity measurements were reported from: Janeway, Travers et al. 2005 for FcαRI; Gould, Sutton et al. 2003 for FcεR and FcεRI; and Bruhns, Iannascoli et al. 2009 for the IgG subclasses.

† R131/H131 polymorphic variants, respectively.

†† F158/V158 polymorphic variants, respectively.

ADCC= antibody dependent cellular cytotoxicity; ADCP=antibody dependent cellular phagocytosis

Antibody classes and their subclasses have different biological activity mediated through their Fc regions which are crucial in modulating a variety of cell-mediated immunological responses such as phagocytosis and cellular cytotoxicity regulated through the FcRs on immune cells. These Fc-mediated mechanisms result in the engagement of a variety of immune cells to mount a response against antibody bound target antigens. Harnessing the Fc-FcR interactions is a worthwhile strategy in the development of antibody therapeutics (Woof 2012). This is because antibodies are not only highly specific molecules with favorable pharmacokinetics properties, able to block certain receptor-ligand interactions and trigger Fab-directed signaling even upon engagement with target antigen, but they can also engage immune effector cells to specifically target the destruction of unwanted substances in the body (Table 1.2).

1.1.3 Therapeutic Mechanisms of Action of Monoclonal Antibodies

Because of the specificity of antibodies to their antigens, antibodies have become an important class of drugs used to target specific cells or neutralize and eliminate soluble proteins such as cytokines. Antibodies have been engineered to fight infection, inflammation and cancer (Reichert 2011). There are potentially multiple mechanisms of action of antibody therapeutics (Table 1.3) including blockade of relevant receptors, inhibition of growth factors, inhibition of signaling, virus neutralization, specific delivery of toxins or radioisotopes to tumor cells, and the activation of the immune system to kill targeted cells by mechanisms such as complement dependent cytotoxicity (CDC) and antibody dependent cytotoxicity and phagocytosis (ADCC and ADCP) (Carter 2006). Alternative strategies have

been employed to enhance these mechanisms of action, including the engineering of antibodies or antibody fragments with specificity to more than one antigen to recruit immune cells to the target (bi-specific antibodies), the engineering of differentially glycosylated Fc regions to enhance bioavailability, or the use of peptides to produce anti-idiotypic antibodies in patients to produce human antibodies with Fab regions that mimic antigenic epitopes (Riemer and Jensen-Jarolim 2007; Chames and Baty 2009; Jefferis 2009).

All marketed antibodies are of the IgG class (Reichert 2012) due to favorable pharmacokinetic properties; however, different antibody subclasses have been employed therapeutically, such IgG1, IgG2 and IgG4 depending of the desired role of Fc-mediated mechanisms of action. Antibodies of the IgG1 subclass generally have higher affinity to FcγRs and it is largely thought that this subclass can mediate the most potent effector functions (Bruggemann, Williams et al. 1987; Bruhns, Iannascoli et al. 2009). In contrast to the other IgG subclasses, the IgG4 antibody subclass is generally thought to have overall weak affinity to FcγRs and weak effector functions and has been employed therapeutically when antibody Fc-mediator effector functions are not desirable (Bruggemann, Williams et al. 1987; Bruhns, Iannascoli et al. 2009). There has been some exploration of the use of antibodies of alternate Ig classes such as IgA and IgE (reviewed further in Section 1.3.3.1) in cancer immunotherapy to enhance Fc-mediated effector functions by the engagement of alternate FcRs on immune cells such as FcαRI, FcεRI, and CD23 (Table 1.2) (Woof 2012).

Table 1.3 Mechanisms of Action of Marketed Monoclonal Antibodies in Several Disease Indications

Target	Isotype	Monoclonal Antibody/conjugate	Mechanisms of action
Oncology			
CD20	IgG1	Rituximab (Mabthera®)	ADCC, direct induction of apoptosis, and CDC
	IgG1	Ofatumumab (Arzerra®)	ADCC and CDC
	IgG1	^{90}Y-labelled Ibritumomab/ tiuxetan (Zevalin®)	Delivery of radioisotope
	IgG2a (murine)	^{131}I-labelled Tositumomab (Bexxar®)	ADCC, direct induction of apoptosis and delivery of radioisotope
CD30	IgG1	Brentuximab vedotin (Adcetris®)	Delivery of toxin
CD33	IgG4	Gemtuzumab/ ozogamicin (Mylotarg®)*	Delivery of toxin
CD52	IgG1	Alemtuzumab (Campath®)	Direct induction of apoptosis and CDC
EGFR	IgG1	Cetuximab (Erbitux®)	Inhibition of EGFR signaling and ADCC
	IgG2	Panitumumab (Vectibix®)	Inhibition of EGFR signaling and ADCC
HER2	IgG1	Trastuzumab (Herceptin®)	Inhibition of HER2 signaling and ADCC
	IgG1	Pertuzumab (Perjeta®)	Inhibition of HER2 signaling and ADCC
VEGF	IgG1	Bevacizumab (Avastin®)	Inhibition of VEGF signaling
CTLA-4	IgG1	Ipilimumab (Yervoy®)	Inhibition of CTLA-4 signaling
Inflammation			
BAFF	IgG1	Belimumab (Benlysta®)	Inhibition of B cell activator
IgE	IgG1	Omalizumab (Xolair®)	Blockade of free IgE receptor binding
IL-12/IL-23	IgG1	Ustekinumab (Stelara®)	Receptor blockade
IL-1B	IgG1	Canakinumab (Ilaris®)	Receptor blockade
IL-6R	IgG1	Tocilizumab (Actemra®)	Receptor blockade
RANK-L	IgG2	Denosumab (Prolia®)	Inhibition of ligand binding
TNF-α	IgG1 IgG1 IgG1 IgG1	Infliximab (Remicade®); Adalimumab (Humira®); Certolizumab pegol (Cimzia®); Golimumab (Simponi®)	Receptor blockade
α4β integrin	IgG4	Natalizumab (Tysabri®)	Blockade of immune cell adhesion
Infectious disease			
RSV	IgG1	Palivizumab (Synagis®)	Pathogen neutralization
Cardiovascular			
gp iib/iiia	IgG1	Abciximab (ReoPro®)	Inhibitor of platelet aggregation
Transplantation			
CD25	IgG1	Basiliximab (Simulect®)	Inhibition of T cell proliferation and B cell activation
Hematological disorders			
C5	IgG2	Eculizumab (Soliris®)	Complement blockade

* Withdrawn from use. BAFF= B cell activating factor; CTLA-4= cytotoxic lymphocyte antigen 4; C5= complement component 5; EGFR= epidermal growth factor receptor; gp=glycoprotein, HER2= human epidermal growth factor receptor 2; IL=interleukin; RANKL= receptor activator of nuclear factor kappa-B; RSV=respiratory syncytial virus; TNF= tumor necrosis factor; VEGF= vascular endothelial growth factor. Table adapted from Reichert 2012.

Monoclonal antibodies can specifically target the destruction of cancer cells which over-express certain tumor associated antigens (TAAs) such as Her2/neu or EGFR in breast and colorectal cancer, respectively. Antibodies with specificity to these cell surface TAAs, once bound to cancer cells, can directly inhibit antigen function through their Fab regions and/or can engage FcRs on immune effector cells through their Fc regions thereby recruiting the immune system to specifically kill these cancer cells (Hudis 2007; Nimmerjahn and Ravetch 2007; Nimmerjahn and Ravetch 2008). Antibodies can also be conjugated to other drugs such as immunotoxins, radioisotopes or enzymes followed by a prodrug in the case of antibody-directed enzyme prodrug therapy, to specifically deliver these agents to targeted cancer cells (Mayer, Francis et al. 2006; Junutula, Flagella et al. 2010; FitzGerald, Wayne et al. 2011).

As cancer therapeutics, the binding of antibodies to cell surface TAAs can result in the recruitment of immune effector cells and the crosslinking of activating FcRs on these immune cells to mediate ADCC and ADCP (Figure 1.2). The engagement of FcγRs on immune cells by IgG tumor-specific antibodies to mediate ADCC of tumor cells has emerged as an important mechanism of action for some monoclonal antibodies (Clynes, Takechi et al. 1998; Carter 2006). The importance of the Fc-mediated mechanisms of action of antibody therapeutics to engage human host immunity has also become clear by recent studies evaluating the correlations between a patient's polymorphisms in FcγRs and the clinical efficacy of antibodies (Cartron, Dacheux et al. 2002; Zhang, Gordon et al. 2007; Musolino, Naldi et al. 2008). Fc-mediated functions of antibodies are gaining interest in light of more recent insight into their important contribution to the mechanisms of action of some antibodies, such as Trastuzumab (Spector and Blackwell 2009).

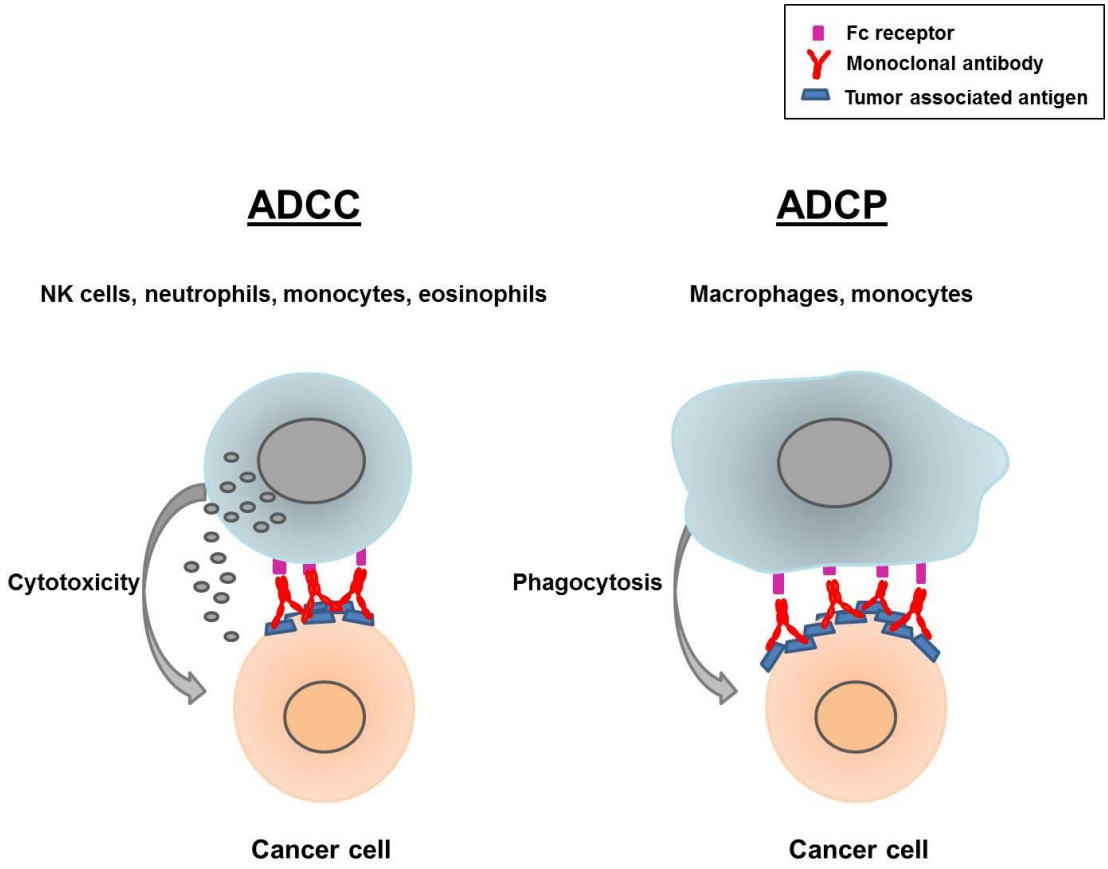


Figure 1.2 Fc-mediated cellular mechanisms of action of antibodies directed against tumor associated antigens. Antibodies targeting TAAs can interact with FcRs expressed on the surface of innate immune cells, resulting in the targeted destruction of antibody coated tumor cells. This antibody Fc-mediated tumor cell killing can be a result of ADCC or ADCP. Tumor cell ADCC can be mediated by NK cells, neutrophils, monocytes and eosinophils by the release of cytotoxic granules such as granzymes, perforins, along with the release of inflammatory mediators such as TNFα and IFN-γ resulting in tumor cell apoptosis. Antibodies recognizing TAA can also mediate ADCP through interactions of FcRs on phagocytes such as macrophages and monocytes resulting in the phagocytosis of these antibody coated tumor cells.

1.1.4 Monoclonal Antibodies for the Treatment of Cancer

Monoclonal antibodies have emerged as an important class of drugs in oncology over the last twenty years, with 13 monoclonal antibodies having been approved for use in oncology (Weiner, Surana et al. 2010; Lipson and Drake 2011; Reichert 2011). Approved monoclonal antibodies are currently used to treat a variety of hematological malignancies such as leukemia and lymphoma along with more recently developed antibodies that are available for the treatment of some solid tumors such breast, lung, colorectal cancers and most recently melanoma (Weiner, Surana et al. 2010; Lipson and Drake 2011). These approved antibodies target TAAs which include hematopoietic differentiation antigens (CD20, CD30, CD33, and CD52), human epidermal growth factor receptor (HER) 2 (HER2; erbB2/neu; Her2/neu) or HER1 (human epidermal growth factor receptor [EGFR]), vascular endothelial growth factor (VEGF), which contributes to angiogenesis in the tumor vasculature, and cytotoxic lymphocyte antigen 4 (CTLA-4) which regulates T cell responses (Table 1.4). Currently, antibody therapeutics for the treatment of cancer encompasses the targeting of only 8 antigens (Table 1.4); however, there are several monoclonal antibodies being evaluated in clinical studies that target new groups of TAAs. Examples of such antibodies (Table 1.5) include those against glycoproteins and carbohydrates expressed by solid tumors, stromal and extracellular matrix (ECM) antigens, and new anti-angiogenic targets (Reichert 2011; Scott, Wolchok et al. 2012).

Table 1.4 Monoclonal Antibodies Approved for Use in Oncology

Target	Monoclonal antibody/conjugate	Antibody isotype	FDA-approved indication	Year of approval in EU (US)	Mechanisms of action
Hematological malignancies					
CD20	Rituximab (Mabthera®)	Chimeric IgG1	Non-Hodgkin's lymphoma and chronic lymphocytic leukemia	1998 (1997)	ADCC, direct induction of apoptosis, and CDC
	Ofatumumab (Arzerra®)	Human IgG1	Chronic lymphocytic leukemia	2010 (2009)	ADCC and CDC
	^{90}Y-labelled Ibritumomab/ tiuxetan (Zevalin®)	Murine IgG1	Non-Hodgkin's lymphoma	2004 (2002)	Delivery of radioisotope
	^{131}I-labelled Tositumomab (Bexxar®)	Murine IgG2a	Non-Hodgkin's lymphoma	NA (2003)	ADCC, direct induction of apoptosis and delivery of radioisotope
CD30	Brentuximab vedotin (Adcetris®)	Chimeric IgG1	Acute myeloid leukemia	In review (2011)	Delivery of toxin
CD33	Gemtuzumab/ ozogamicin (Mylotarg®)*	Humanized IgG4 immunotoxin	Acute myelogenous leukemia	NA (2000)	Delivery of toxin
CD52	Alemtuzumab (Campath®)	Humanized IgG1	B cell chronic lymphocytic leukemia	2001 (2001)	Direct induction of apoptosis and CDC
Solid malignancies					
EGFR	Cetuximab (Erbitux®)	Chimeric IgG1	SCCHN & colorectal cancer	2004 (2004)	Inhibition of EGFR signaling and ADCC
	Panitumumab (Vectibix®)	Human IgG2	SCCHN & colorectal cancer	2007 (2006)	Inhibition of EGFR signaling and ADCC
HER2	Trastuzumab (Herceptin®)	Humanized IgG1	Breast cancer	2004 (2004)	Inhibition of HER2 signaling and ADCC
	Pertuzumab (Perjeta®)	Humanized IgG1	Breast cancer	In review (2012)	Inhibition of HER2 signaling and ADCC
VEGF	Bevacizumab (Avastin®)	Humanized IgG1	Colon cancer	2007 (2006)	Inhibition of VEGF signaling
CTLA-4	Ipilimumab (Yervoy®)	Human IgG1	Melanoma	2011 (2011)	Inhibition of CTLA-4 signaling

*Withdrawn from use; SCCHN= squamous cell carcinoma of the head and neck; NA= not approved; FDA= Food & Drug Administration
Table adapted from Scott & Wolchok 2012 and Reichert 2011.

Table 1.5 Selected Antibodies against Tumor Associated Antigens in Clinical Development

Antigen category	Targeted antigen	Examples of therapeutic mAbs	Examples of antigen expressing tumor types
Glycoproteins	EpCAM	IGN101 and Adecatumuab	Epithelial tumors (breast, colon and lung)
	CEA	Labetuzumab	Breast, colon and lung tumor
	gpA33	HuA33	Colorectal carcinoma
	Mucins	Pemtumomab and Oregovomab	Breast, colon, lung and ovarian tumors
	PSMA	J591	Prostrate carcinoma
	CAIX	Girentuximab	Renal cell carcinoma
	TAG-72	Minretumomab	Breast, colon and lung tumors
	FRα	MOv18 and Farletuzumab	Ovarian tumors
Carbohydrates	Lewis Y	Hu3S193 and IgN311	Breast, colon, lung and prostate tumors
Growth and differentiation signaling	EGFR	Nimotuzumab and 806	Glioma, lung, breast, colon, and head and neck tumors
	HER3	MM-121	Breast, colon, lung, ovarian and prostate, tumors
	HGFR	Rilotumumab, Onartuzumab and SCH 900105	Breast, colorectal, ovarian and lung tumors
	IGF1R	AVE1642, IMC-A12, MK-0646, R1507 and CP 751871	Glioma, lung, breast, head and neck, prostate and thyroid cancer
	EPHA3	KB004 and IIIA4	Lung, kidney and colon tumors, melanoma, glioma and hematological malignancies
	TRAILR1	Mapatumumab (HGS-ETR1)	Colon, lung and pancreas tumors and hematological malignancies
	TRAILR2	HGS-ETR2 and CS-1008	Colon, lung and pancreas tumors and hematological malignancies
Stromal and extracellular matrix antigens	FAP	Sibrotuzumab and F19	Colon, breast, lung, pancreas, and head and neck tumors
	Tenascin	81C6	Glioma, breast and prostate tumors
Targets of anti-angiogenic mAbs	VEGFR	IM-2C6 and CDP791	Epithelium-derived solid tumors
	Integrin αVβ3	Etaracizumab	Tumor vasculature
	Integrin α5β1	Volociximab	Tumor vasculature

CAIX= carbonic anhydrase IX; CEA= carcinoembryonic antigen; EpCAM= epithelial cell adhesion molecule; EPHA3= ephrin receptor A3; FAP= fibroblast activation protein; gpA33= glycoprotein A33; FRα= folate receptor alpha; HGFR= hepatocyte growth factor receptor; IGF1R= insulin-like growth factor 1 receptor; mAbs= monoclonal antibodies; PSMA= prostate-specific membrane antigen; RANKL= receptor activator of nuclear factor-κB ligand; TAG-72= tumor-associated glycoprotein 72; TRAILR= tumor necrosis factor-related apoptosis-inducing ligand receptor; VEGFR= VEGF receptor. Table reproduced in part from Scott, Wolchok et al. 2012.

Antibodies can afford some advantages over small molecule drugs for the treatment of cancer and other diseases such as their potential for low toxicity, improved pharmacokinetics, and target specificity (Weiner and Borghaei 2006). However, antibodies as cancer therapeutics can also have limitations such as poor tumor penetration, poor oral bioavailability, and are expensive to manufacture. The first FDA approved antibodies in the US were for the treatment of hematological malignances, and monoclonal antibodies have also met considerable success in the treatment of solid tumors in combination with chemotherapy with the approval of Trastuzumab and other EGFR antibodies, such as Cexitumab and Panitumumab, for the treatment of breast and colorectal cancer, respectively (Romond, Perez et al. 2005; Jonker, O'Callaghan et al. 2007). More recently, the monoclonal antibody Ipilimumab has demonstrated a meaningful achievement in the treatment of metastatic melanoma, and it is the first antibody therapy that functions by an immunomodulatory mechanism (reviewed further in Section 1.2.1). While these antibodies have laid the foundations for antibody-based therapies for the treatment of solid tumors, future successes may lie in optimizing antibody Fc structure to enhance anti-tumor responses in the tumor microenvironments along with further understanding of the interaction of the host immune system with cancer cells (Kubota, Niwa et al. 2009; Scott, Wolchok et al. 2012).

1.1.5 Monoclonal Antibody Production

Monoclonal antibodies were coined “magic bullets” by Paul Ehlrich in the early 20th century, soon after their discovery. The immediate clinical application of antibodies as passive immunotherapies was hindered due to limitations around their production. Technological improvements in the engineering of antibodies over the last forty years have enabled the emergence of monoclonal antibodies as a relevant class of drug with demonstrated clinical utility in the treatment of many diseases.

1.1.5.1 Historical Perspectives on the Production of Antibody Therapeutics

In 1975, the development of hybridoma technology by Kohler and Milstein allowed for the production of monoclonal antibodies from laboratory generated cell lines, which was a crucial step in the development monoclonal antibodies as therapeutics. Hybridoma technology, which fuses splenocytes from immunized mice with murine myeloma cells, resulted in the production of a hybridoma cell line capable of producing antibodies of a desired specificity (Kohler and Milstein 1975). This work was a major breakthrough in the scientific community, permitting the production of antibodies in unlimited amounts to serve as valuable research tools; although without any further manipulation, these antibodies had limited clinical therapeutic utility being of murine origin. The murine antibodies produced by hybridoma were immunogenic in humans, resulting in the production of human anti-murine antibodies, fast clearance, and had poor ability to engage human immune effector responses (Shawler, Bartholomew et al. 1985; Ober, Radu et al. 2001).

Following the invention of hybridoma technology, there have been numerous efforts leading to the successful production of more human/fully human antibodies over the last forty years (Figure 1.3). The advancement of strategies to engineer fully human antibodies has resulted in the reduced concerns of immunogenicity along with the potentially greater efficacy of fully human antibodies which have superior pharmacokinetic properties compared to mouse antibodies because of FcRn binding (Ober, Radu et al. 2001). Additionally, antibodies with fully human Fc regions may optimally engage human Fc receptors on immune cells to mediate cellular mechanisms of action against tumor cells such as ADCC and ADCP. As a first step towards the creation of fully human antibodies, recombinant DNA technologies were employed to join murine variable regions to human constant regions in order to produce chimeric antibodies (Sahagan, Dorai et al. 1986). Further genetic engineering of these chimeric antibodies resulted in the production of humanized antibodies containing only murine CDRs (Carter, Presta et al. 1992). And more recently, techniques have emerged to produce fully human monoclonal antibodies using either *in vivo* or *in vitro* approaches, which have included phage display technologies and the creation of transgenic mice with humoral immune systems such as the Xenomouse® (Vaughan, Osbourn et al. 1998).

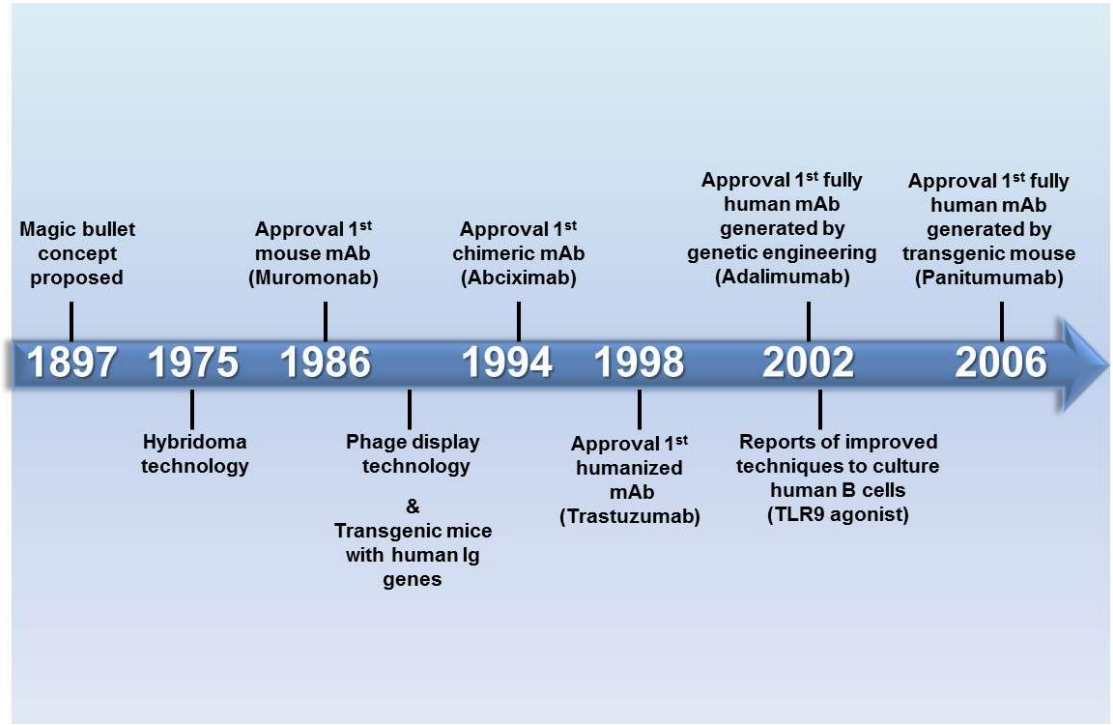


Figure 1.3 Timeline of the progress of monoclonal antibody production.
Following the concept of antibodies as "magic bullets" there have been many efforts to manufacture mAbs as drugs for the treatment of diseases such as infection, inflammation and cancer. The first marketed antibody approved by the US FDA was a fully murine antibody, Muromonab, produced using hybridoma technology. Due to production of human anti-mouse antibodies following the administration of mouse mAbs in humans, efforts over the next 20 years focused on producing more fully human mAbs. These first efforts resulted in the production of chimeric murine-human antibodies containing murine variable regions and human constant regions, followed by humanized antibodies containing only murine CDRs. Next, the production of fully human mAbs was achieved either by the application of antibody phage display technology, or the use of transgenic mice with human immunoglobulin genes. Also of note is the more recent development of methods to improve the culture of human B cells.

The generation of fully human therapeutic antibodies against a desired antigen has been achieved *in vitro* by the use of antibody phage display technology. This technology utilizes large sized libraries of heavy and light chain encoding genes which are cloned and then displayed on the surface of cells such as bacteriophages to pan for reactivity against a particular antigen (McCafferty, Griffiths et al. 1990; Barbas 1995). Following the selection of antibody fragments with desired specificity, these Fab or single-chain fragments of the variable region (scFv) can then be cloned into full sized human IgG antibodies. This approach can be applied to generate monoclonal antibodies against TAAs through the use of complementary deoxyribonucleic acid (cDNA) libraries generated from mice following immunization (Chester, Begent et al. 1994) or through the use of synthetic or human cDNA combinatorial libraries (Mao, Gao et al. 1999). One advantage of this approach is that it can be independent from the mammalian immune system and possibly could avoid issues such as immune tolerance and epitope dominance and can lead to the generation of high number of antibody fragments with specificity to a given antigen. Also, because the variable region genes are already cloned, this approach does not necessarily require laborious techniques such as humanization. In some cases, disadvantages of the production of antibodies by phage display can include the need to convert antibody fragments to the whole IgG format, the random pairing of heavy and light chains, and that some antibodies can have low affinity to antigen and thus require affinity maturation and lead optimization, processes that can take months. However, new technologies are emerging to circumvent these limitations, such as the construction of novel libraries with unique combinations of Ig sequence from human donor and synthetic diversity (Hoet, Cohen et al. 2005) or the coupling *in*

vitro of somatic hypermutation with mammalian cell display resulting in the immediate generation of affinity matured antibodies (Bowers, Horlick et al. 2011).

An *in vivo* approach to generate fully human therapeutic antibodies has been the creation of transgenic mice expressing human immunoglobulin genes. Transgenic mice such as the Xenomouse® have entirely human immunoglobulin loci in their germ lines and are deficient in the production of mouse Ig (Jakobovits, Amado et al. 2007). Because these transgenic animals are capable of producing antigen-specific B cell responses and undergoing mammalian immune processes key to generating antibody diversity such as somatic hypermutation, the resulting antibodies are often of high affinity (Green, Hardy et al. 1994; Lonberg, Taylor et al. 1994). These mice can rapidly generate fully human antibodies following immunization with target antigen, unlike antibody phage display which may require lead optimization and conversion into IgG format (Lonberg 2008). There could be limitations to this approach with regards to the generation of antibodies against highly conserved antigens which may not be produced due to mechanisms of tolerance and also antibodies produced by these transgenic mice may be against dominant antigenic epitopes rather than those that inhibit antigen function (Chames, Van Regenmortel et al. 2009).

The production of fully human antibodies either by transgenic mice with human antibody genes or phage display technologies has been successfully translated into clinical use in the last ten years (Figure 1.3). The first marketed antibody therapeutic with a fully human IgG structure was Adalimumab, approved for the treatment of rheumatoid arthritis in 2002, which was produced using antibody phage display technology (Lonberg 2008). The first fully human monoclonal antibody created from transgenic mice was Panitumumab, approved for the

treatment of colorectal cancer in 2007 (Jakobovits, Amado et al. 2007). Both the use of Xenomouse® and antibody phage display represent technologies capable of generating fully human antibodies, but have limitations in that they can require the use of proprietary animals such as the Xenomouse® or of bacterial cells to express mammalian proteins which involve the lengthy process of *in vitro* antibody affinity maturation and further engineering into IgG format.

The above strategies employing immunization or surface display approaches to create fully human antibodies require the use of known antigens either for immunization (transgenic mice) or for selection of specific variable regions (antibody phage display). Recently, there has been some renewed interest in the identification and generation of fully human antibodies by the immortalization of patient B cells, a strategy which presents the unique potential to select naturally affinity-matured antibodies with matched heavy and light chains, without the requirement of a known antigen or the more lengthy process of *in vitro* maturation. Human B cell immortalization presents an additional methodology to produce human antibodies which could lead to the discovery of novel antibodies, possibly against undiscovered antigens.

1.1.5.2 Strategies to Discover Human Antibodies from Patient B cells

An alternate strategy for the generation of novel antibodies to those previously described such as hybridoma and phage display, is the immortalization of human B cells following exposure to pathogens. The key advantages of this approach are that it does not require antigen for the production of antibodies, or necessarily require the use of additional host cell lines to express antibodies for antibody characterization, and results in the immediate production of human antibodies. This approach also has the potential to reveal antibodies of novel specificity by studying natural human antibody responses following the exposure to foreign pathogens. The immortalization of human B cells into antibody-secreting cell lines has been primarily achieved by the co-incubation with Epstein-Barr virus (EBV), a lymphotrophic herpes virus that infects human B cells resulting in B cell proliferation (Miller and Lipman 1973). EBV has been demonstrated to 'immortalize' human B cells in culture to create lymphoblast cell lines (Steinitz, Klein et al. 1977) and this method has been widely used. Unfortunately, the realization of this approach to generate fully human monoclonal antibodies has been limited by the historically low efficiency rates of B cells, with reported rates of EBV transformation being as low 3% of B cells (Sugden and Mark 1977). Additionally, B cells cultured *ex vivo* have historically not survived for long periods in culture or secreted acceptable amounts of antibodies for adequate study. These difficulties have hindered the widespread application of this technique in the past to study the breadth of natural antibody responses from human B cells and to select for promising antigen-specific B cell clones.

There have been more recent improvements to the culturing of human B cells such as the addition of polyclonal B cell activators to enhance EBV transformation efficiencies or the genetic programing of B cells. In 2004, Traggiai and colleagues found that the addition of a polyclonal B cell activator, a CpG oligonucleotide (CpG ODN 2006, CpG ODN or CpG 2006), which is a toll-like receptor (TLR) 9 agonist (Figure 1.4), resulted in the improved efficiency rates of memory B cell transformation ranging from 30 to 100% (Traggiai, Becker et al. 2004). This methodology preferentially activates and expands memory B cells and the production of Igs in culture through the activation of TLR9 receptors, which are more highly expressed on memory B cells compared to naïve B cells and do not require BCR triggering for activation in culture (Bernasconi, Traggiai et al. 2002; Bernasconi, Onai et al. 2003). A second example of a recent improvement to the culturing of human B cells is the retroviral introduction of genes into B cells, which increases cell proliferation in culture. Kwakkenbos and colleagues found that the introduction of genes for B cell lymphoma-6 and xL proteins which have been implicated in the inhibition of terminal B cell differentiation *in vitro* and blocking of apoptosis, respectively, along with the co-culture of CD40 ligand and IL-21, increased the *in vitro* expansion of B cells (Kwakkenbos, Diehl et al. 2010). This technique resulted in the simultaneous expression of BCR and secretion of antibodies which allowed for the screening of B cells with desired antigen specificity (Kwakkenbos, Diehl et al. 2010). These approaches have been successfully applied to discover novel neutralizing antibodies from patients following exposure to pathogens.

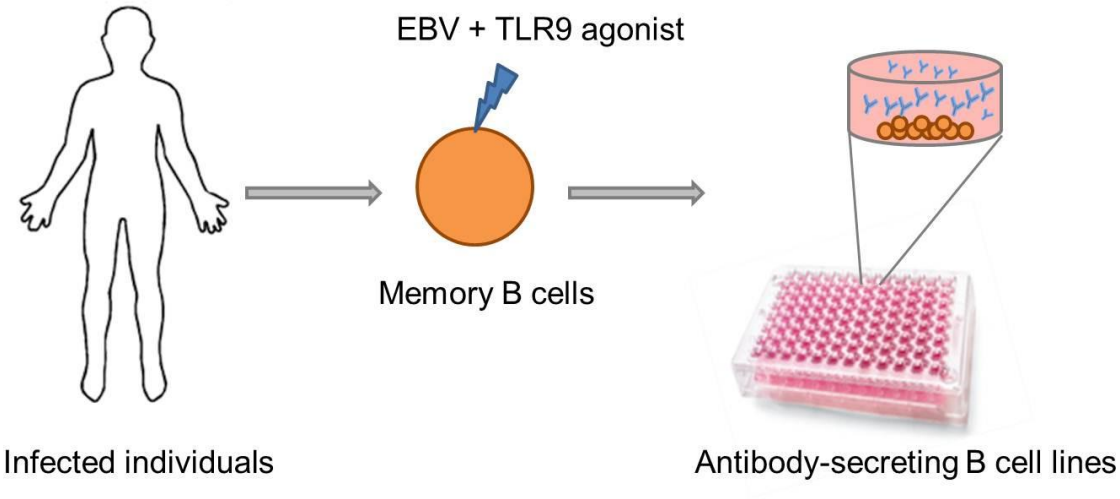


Figure 1.4 Generation of antibody-secreting cell lines from humans. Following the isolation of B cells from patients, antibody-secreting B cell cultures can be rapidly generated through the co-culture of B cells with EBV and the TLR9 agonist, CpG 2006, along with irradiated autologous PBMCs. Antibodies secreted from these patient-derived B cell lines can then be further studied to elucidate immunological memory responses, such as those following infection with a virus.

Improvements in the culturing of human B cells have resulted in the generation of neutralizing monoclonal antibodies in infectious diseases. Traggiai and colleagues identified 35 neutralizing monoclonal antibodies to the SARS corona-virus from one infected individual, illustrating their technique was a rapid and efficient approach to the discovery of monoclonal antibodies against a virus (Traggiai, Becker et al. 2004). Others have applied and slightly modified these techniques to identify antibodies against cytomegalovirus (Funaro, Gribaudo et al. 2008). And Kwakkenbos and colleagues demonstrated that their genetic reprograming techniques could produce monoclonal antibodies capable of neutralizing the respiratory syncytial virus. Importantly, antibody repertoires from patient memory B cells following exposure to a virus provide new understanding of antibody responses to infectious diseases such as influenza (Wrammert, Smith et

al. 2008; Li, Chiu et al. 2012) and Human Immunodeficiency virus (HIV) (Scheid, Mouquet et al. 2009) and can inform the design of efficacious vaccines by identifying antigenic epitopes. In light of these recent advancements in the transformation of human B cells, the study of antibody repertoires following the development of infectious diseases in individuals provides an alternate strategy in the discovery of fully human monoclonal antibodies, and deserves further consideration in more disease settings.

Harnessing the antibody repertoire of individuals with cancer through the transformation and immortalization of patient B cells could provide an alternate strategy in the discovery of antibodies against cell surface TAAs. Naturally occurring antibody responses to cancer antigens have been described in the serum, circulating memory B cells, and tumor-infiltrating B cells of individuals with melanoma (reviewed in Section 1.3.1) and other cancers such as breast and lung cancers (Coronella-Wood and Hersh 2003; Kotlan, Simsa et al. 2005; Mizukami, Hanagiri et al. 2005). Most of these studies have centered on humoral immune responses to melanoma because it is widely thought to be a prototypic immunogenic cancer. Yet prior to the work done in this thesis, there are no reports of applying these reported improvements for *ex vivo* B cell activation to evaluate antibodies produced in individuals in response to cancer antigens.[1] It is hypothesized here that application of the B cell activator described by Traggiai et al., namely CpG 2006, to the EBV immortalization of human B cells from melanoma patients could yield an efficient route to identify and generate fully human antibodies against TAAs.

[1] Results described in this thesis evaluating these methods have been previously published elsewhere (Gilbert, Karagiannis et al. 2011).

Historically, there have been several attempts to derive melanoma-reactive antibodies from EBV transformed patient-derived peripheral blood and tumor-resident B cells. These efforts have yielded a few antibodies of the IgM and IgG class (Kirkwood and Robinson 1990; Punt, Barbuto et al. 1994). A major technical obstacle of these studies was reported to be loss of antibody production from long term B cell cultures (Kirkwood and Robinson 1990; Yeilding, Gerstner et al. 1992), providing justification of applying improved methodologies to increase human B cell transformation *in vitro* in order to allow for a more comprehensive study of the antibody repertoires in melanoma patients. Studying the antibody response of individuals with melanoma could possibly afford multiple advantages in the discovery of novel antibodies: antigens do not need to be known a priori; high affinity matured human antibodies can be identified; clinically relevant clones can be selected by studying patients' antibody responses against melanoma cells; and human B cell cultures could provide an immediate source of antibodies with which to perform early *in vitro* characterizations.

Important to the attainment of tumor-specific antibodies from the B cells of cancer patients is the development of *in vitro* screening assays to identify antibodies with reactivity to TAAs. This poses a challenge in that few cell surface antigens have been described for melanoma and are readily commercially available in recombinant form. For these reasons, the identification of melanoma-reactive antibodies from patient B cells has primarily been done against tumor cell lines which are readily available (Chen, Güre et al. 1998; Carter, Smith et al. 2004) and do not require the identification or production of TAAs. Since cancer is widely thought to be heterogeneous and cell lines may differentially express antigens, the evaluation of multiple cell lines would be crucial in this screening approach. While

it could be assumed that cell lines may have altered protein expression after several generations in culture, some studies have shown antigens in cell lines correspond to antigen expression on freshly isolated tumors making them a suitable starting point (Degiovanni, Hainaut et al. 1990; Wistuba, David Bryant et al. 1999). Furthermore, the identification of tumor-reactive antibodies from patients has been performed using cell lines with techniques such as hemadorsption assays, immunohistochemistry (IHC), immunocytochemistry (ICC) using cytospins, immunoblots, enzyme-linked immunoabsorbant assay (ELISA), and fluorometric microvolume assay technology (Carey, Takahashi et al. 1976; Yeilding, Gerstner et al. 1992; Punt, Barbuto et al. 1994; Miraglia, Swartzman et al. 1999; Carter, Smith et al. 2004). Therefore, essential to performing a comprehensive screening of antibodies from many individuals would be the generation of an inexpensive, medium-throughput cell-based assay for screening using available resources to aide in the discovery of novel antibodies against TAAs present in patients with cancer. The design of such an assay was a primary goal of this thesis, to permit for the identification and characterization of melanoma-reactive antibodies secreted from the B cells of individuals with melanoma.

1.2 Melanoma

Malignant melanoma is one of the most lethal forms of skin cancer; while it accounts for less than 5% of skin cancer cases it is responsible the majority of skin cancer related deaths, and incidence rates are sharply rising world-wide compared to other cancers (Lens and Dawes 2004; ACS 2012). Melanoma arises from the

unregulated growth of melanocytes, pigmented cells found primarily in the skin and other epithelial surfaces such as the eye. Melanocytes originate from highly motile neural crest progenitors and melanoma can be a highly invasive cancer. If diagnosed early, and localized in the skin, 98% of these individuals can be cured by surgical resection (ACS 2012). However once metastatic, patient prognosis becomes increasingly poor with disease progression, and patients diagnosed with Stage IV melanoma have reported median survival rates of 6 to 10 months and 5-year survival rates between 5 and 22% (Cummins, Cummins et al. 2006; Hodi, O'Day et al. 2010; ACS 2012; CRUK 2012). Historically, melanoma in its advanced stages has been proven highly resistant to treatments such as chemotherapy (Soengas and Lowe 2003).

Melanoma is widely accepted to be the prototypic immunogenic tumor, and this notion has been supported by reports of partial or complete regression of melanoma lesions, reported cases of spontaneous remissions in patients, along with evidence of tumor-specific T and B cell responses (Stockert, Jäger et al. 1998; Lee, Yee et al. 1999; Kalialis, Drzewiecki et al. 2009). This could be viewed as a paradox when considering that malignant melanoma has historically been highly resistant to treatment in its advanced stages, even with the use of therapies aimed at enhancing host immunity. The immunogenic nature of melanoma coupled with the low survival rates of patients with malignant disease exemplify the complexity of the relationship between the host immune system and cancer, discussed further in Section 1.3. Examination of the immune response in melanoma provides opportunities to elucidate immunomodulatory mechanisms and the potential to harness components of the immune system for therapeutic purposes.

1.2.1 Therapy for Metastatic Melanoma

Until recently, only a few therapies were approved for the treatment of malignant melanoma by the US FDA (Table 1.6), and these therapies have had limited clinical results. Dacarbazine, a chemotherapeutic agent, has been used as a standard therapy in melanoma for decades; however, it has only shown short-lived clinical responses without evidence for improvement of patient survival (Hill, Krementz et al. 1984). The second type of therapy used in melanoma has been immunotherapy, through the use of the immune activating cytokines high dose interleukin (IL)-2 or interferon (IFN) α2b. IL-2 is a cytokine that increases the proliferation of NK, B and T cells and upon administration to patients it can result in the expansion of these lymphocytes thus increasing their effector function against tumor cells (Waldmann 2006). The administration of high doses of IL-2 has met with some success in the clinic, but unfortunately it has been limited to some subgroups of patients and it is associated with high rates of toxicity (Atkins, Lotze et al. 1999). Another immunotherapy that has been used to treat high risk melanoma is IFNα2b, a highly pleiotropic cytokine thought to have anti-proliferative, apoptotic, and anti-angiogenic properties along with the promotion of a host anti-tumor response brought on by the stimulation of cytotoxic cells such NK cells, and the enhancement of dendritic cell presentation to prime tumor-specific T cells (Kirkwood, Tarhini et al. 2008; Diamond, Kinder et al. 2011). It has been hypothesized that the use of this cytokine can induce T helper cell subset 1 (Th1) responses against the tumor in micrometastases thought to associated with less established immune tolerance (Tarhini and Kirkwood 2009). Clinical studies administering high doses of IFNα2b have demonstrated a reduction in the reoccurrence of melanoma, however a clear impact of IFNα2b on overall patient

survival rates has not been demonstrated (Wheatley, Ives et al. 2003). IFNα2b and IL-2 have both demonstrated clinical utility for the treatment of some patient groups since their FDA approval in 1995 and 1998, respectively. However, the overall lack of effective therapies to treat larger groups of patients remained and resulted in the generation of considerable scientific interest in the discovery and development of new therapeutics aimed at improving patient outcomes.

Table 1.6 US FDA Approved Drugs for the Treatment of Malignant Melanoma

Drug (brand name)	Specificity	Class	Mechanism of action	Year of approval
Dacarbazine (DTIC-Dome®)	Non-specific	Chemotherapy	Alkylating agent leading to DNA damage	1975
IFNα2b† (INTRON® A)	IFNα receptor 1 and 2	Immunotherapy (cytokine)	Multifunctioning immunomodulatory cytokine, anti-angiogenesis, anti-proliferation and apoptosis	1995
High dose IL-2 (Aldesleukin, Proleukin®)	IL-2 receptor expressed on lymphocytes	Immunotherapy (cytokine)	Immune activating, increases proliferation of immune cells such as NK, B, and T cells	1998
Ipilimumab (Yervoy®)	CTLA-4 expressed on T cells	Immunotherapy (mAb)	Activates immune system by enhancing T cell activation	2011
Verumafenib (Zelboraf®)	BRAF V600E, mutated form of BRAF protein	Small molecule inhibitor	Blocks mitogen-activated protein kinase pathway reducing proliferation of melanoma cells carrying mutation	2011

† A pegylated version of IFNα2b was approved in 2011 with enhanced efficacy and half-life.

The recent approval of two new drugs in 2011 by the US FDA has brought much promise in the treatment of metastatic melanoma. The first drug, Vemurafenib (PLX4032 or Zelboraf®) is a small molecule kinase inhibitor which selectively targets a mutant form of the BRAF protein, namely BRAF V600E, which has been detected in 66% of patients with malignant melanoma (Davies, Bignell et al. 2002).

This inhibitor can selectively block the mitogen-activated protein kinase (MAPK) pathway, thereby reducing the proliferation of melanoma cells carrying this BRAF mutation. Application of this agent has shown complete or partial tumor regression in a Phase I trial (Flaherty, Puzanov et al. 2010). In a Phase II trial, Vemurafenib demonstrated a 50% response rate and median survival rate of 16 months for those individuals with Stage IV melanoma carrying this BRAF V600E mutation (Sosman, Kim et al. 2012). Patients with the BRAF V600E mutation in a Phase III trial were observed to have response rates of 48% for Vemurafenib and 5% for Dacarbazine, with survival rates of 84% and 64% for Vemurafenib and Dacarbazine, respectively, in the 6 months following treatment (Chapman, Hauschild et al. 2011). These clinical data demonstrate the ability of Vermaurafenib to increase the rate of survival and patient responses following treatment.

The second promising drug with recent approval for the treatment of melanoma is Ipilimumab, a monoclonal antibody targeting the high affinity receptor CTLA-4 expressed on T cells. Upon binding to its ligand, CD80 or CD86, CTLA-4 can transmit an inhibitory signal to T cells, and it is hypothesized that the blockade of this receptor may result in the enhancement of T cell-mediated anti-tumor immunity (O'Day, Hamid et al. 2007). The mechanism of action of this antibody is discussed further in Section 1.2.2. In a Phase III study, metastatic melanoma patients had survival rates of approximately 10-11 months when treated with Ipilimumab with or without the gp100 peptide, compared to a survival of rate of 6.4 months for those receiving the peptide vaccine alone (Hodi, O'Day et al. 2010). Further follow up for 177 patients treated with Ipilimumab has shown that median survival rates ranged from 13-16 months and complete response rates ranged

from 6 to 17% for three clinical trials, highlighting the promise of this immunotherapy to sustain tumor regressions (Prieto, Yang et al. 2012). The success of the monoclonal antibody Ipilimumab has been a significant breakthrough for the treatment of malignant melanoma, particularly for those patients diagnosed with metastatic melanoma that do not carry the BRAF V600E mutation and potentially for those patients who relapse following treatment with Vemurafenib. This treatment thus represents a new class of promising cancer immunotherapies aimed at blocking immune inhibitory pathways which can boost the host immune response to cancer.

While Ipilimumab and Vemurafenib are associated with some complete responses and show encouraging results with respect to the prolonged survival of patients, the need for further therapeutic options in melanoma remains, and many on-going efforts are focused on exploring similar or other novel immunotherapeutic approaches.

1.2.2 Immunotherapeutic Approaches for the Treatment of Melanoma

Melanoma is widely thought to be highly immunogenic in nature, supported with clinical observations of spontaneous regression (Bulkley, Cohen et al. 1975; Kalialis, Drzewiecki et al. 2009), the presence of tumor-infiltrating lymphocytes (Clemente, Mihm et al. 1996; Erdag, Schaefer et al. 2012), and clinical responses to immune stimulation such as the polyclonal activating exogenous cytokines IL-2, IFNα2b (Kirkwood, Tarhini et al. 2008). Many therapeutic approaches are aimed at enhancing or modulating these host immune responses by actively (vaccines or mimitopes) or passively (monoclonal antibodies or adoptive cell therapy

approaches) engaging the immune system to produce an anti-tumor response, or in the case of adoptive cell therapies to provide the patient with more potent anti-tumor immune effector cells.

An active immunotherapeutic approach to the treatment of melanoma has been in the development of vaccines. Melanoma vaccines are based on immunizing patients against their own cancers using plasmid DNA or viral vectors, peptides, proteins, tumor cell lysates, and whole cells, including autologous cells. Melanoma vaccines can result in induction of host T cell or humoral immune response against cancer cells. Much work has been done on the development of vaccines for melanoma, however the results of these vaccines have not yet reached profound benefits for large groups of patients (Rosenberg, Yang et al. 2004; Latzka, Gaier et al. 2011; Lacy, Karagiannis et al. 2012). A limitation of active immunotherapies may be the inability to induce a potent enough response in patients to overcome local immune suppression found in metastatic cancers. However, this potentially can be overcome with the development of novel delivery systems such as electroporation for DNA vaccines (Rice, Ottensmeier et al. 2008), or by the enhancement of host lymphocyte responses against the vaccine by the co-administration of immune cell activating cytokines such as IL-2 (Schwartzentruber, Lawson et al. 2011), or through dendritic cell vaccine approaches (Nestle, Alijagic et al. 1998).

Cell-based immunotherapies such as dendritic cell vaccines or adoptive T cell therapies have shown some promise for the treatment of melanoma. The *ex vivo* activation of autologous dendritic cells with peptides or tumor cell lysates to trigger host tumor-specific T cell responses has shown some responses in patients (Nestle, Alijagic et al. 1998). While the widespread application of dendritic cell

vaccines has not been fully realized for melanoma, their evaluation has provided insights into elucidating the powerful role of dendritic cells for active immunotherapeutic approaches, and has paved the way for clinical use of a dendritic cell vaccine for prostate cancer in 2010 (Cheever and Higano 2011).

Adoptive cell therapy approaches, mostly focused on activating T cells, have also warranted much attention. The *ex vivo* expansion and activation of autologous tumor-infiltrating lymphocytes (TILs) has led to patient response rates as high as 50% and tumor regressions in clinical studies comprised of small groups of patients (Dudley, Wunderlich et al. 2005; Besser, Shapira-Frommer et al. 2010). While these studies highlight the merit of this strategy to enhance the host lymphocyte responses to melanoma, practical drawbacks to adoptive cell therapies include the labor intensity, risk of infection, expense of autologous TIL or dendritic cell expansion, and the personalized nature of these treatments, making their widespread use challenging.

Monoclonal antibodies represent a passive immunotherapeutic approach for the treatment of melanoma. As mentioned above, monoclonal antibodies can function in multiple ways, including the activation of immune effector cells to mount an anti-tumor response, blockade of receptor-ligand interactions involved in tumor cell proliferation and mobility, and the blockade of immune checkpoints such as those modulating host immune responses (Carter 2006; Pardoll 2012). As described previously, the first monoclonal antibody for use in the treatment of melanoma, Ipilimumab, has been recently approved. This monoclonal antibody functions by blocking the binding of CTLA-4 expressed on activated T cells to its ligands CD80/CD86 expressed on antigen presenting cells. This results in reversal of inhibitory signals which limit T cell proliferation in response to antigen and

CD80/CD86 stimulation (O'Day, Hamid et al. 2007). Ipilimumab represents the first marketed monoclonal antibody that functions by overcoming host tolerance to cancer (Lipson and Drake 2011).

Other emerging immunomodulatory monoclonal antibodies have shown some clinical promise for the treatment of melanoma, such as those targeting the programmed death 1 (PD-1) receptor. This inhibitory receptor, a tyrosine-based inhibitor motif (ITIM) receptor, is a member of the CTLA-4 protein family and is expressed by activated T cells. Antibodies blocking the binding of PD-1 to its ligand, which is expressed on dendritic cells and activated T and B cells, have been shown to increase the T cell anti-tumor responses in melanoma patients (Brahmer, Drake et al. 2010; Topalian, Hodi et al. 2012). Antibodies targeting the PD-1 receptor may result in a more specific regulation of tumor escape than CTLA-4 blockade since a ligand for PD-1 receptor is also expressed on melanoma cells and the blockade of this receptor in tumors may prevent the inhibition of the regulatory response of tumor cells on T cells (Iwai, Ishida et al. 2002; Hino, Kabashima et al. 2010).

Monoclonal antibodies against TAAs residing on the cell surface may also provide effective therapeutic options for melanoma. Antibodies such as Rituximab and Trastuzumab for treatment of lymphoma and breast cancer, respectively, target antigens over-expressed on the surface of cancer cells, and their demonstrated clinical efficacy along with other antibody therapeutics support the merits of the passive administration of monoclonal antibodies against TAAs. Administration of antibodies against antigens over-expressed in melanoma may result in Fab-mediated non-immunological mechanisms of action against melanoma cells and/or may result in the engagement of the innate immune system to specifically

target melanoma cells by mechanism such as CDC and ADCC/ADCP. Examples of such antibodies include those targeting over-expressed molecules implicated in cell adhesion and the metastatic potential of cells such as tyrosinase-related protein (TYRP) 1, melanoma cell adhesion molecule, and the high molecular weight melanoma associated antigen (HMW-MAA) (Chang, Campoli et al. 2004; Patel, Balderes et al. 2007; Staquicini, Tandle et al. 2008). Thus antibodies against melanoma TAAs may provide a combination of non-immunological mechanisms such as the blockade of signaling pathways involved in cell proliferation, tumor cell adhesion and invasion along with engagement of the innate immune system by Fc-mediated effector functions, resulting in the generation of potent passive immunotherapies specifically directed at melanoma cells.

Antibodies targeting the HMW-MAA are of great potential interest for the treatment of melanoma and constitute a partial focus of this thesis. This antigen represents an ideal antibody target because it resides on the cell surface and has been described to have restricted distribution in normal tissues and have high expression in melanomas (Campoli, Chang et al. 2004). Antibodies against multiple regions of the antigen were discovered over 30 years ago following the immunization of mice with melanoma cells and generation of hybridoma cell lines (Wilson, Imai et al. 1981), and a multitude of antibodies have been produced against this highly immunogenic antigen (Herlyn, Steplewski et al. 1983). Antibodies against HMW-MAA (HMW-MAA antibodies) have revealed key aspects of the function of this TAA (reviewed in the next section) in metastatic processes and have been shown to inhibit the proliferation, migration, spreading, and invasion of melanoma cells (Harper and Reisfeld 1983; de Vries, Keizer et al. 1986; Iida, Meijne et al. 1995). Preclinical studies of this molecule, such as those using

mouse xenograft models of melanoma, demonstrated that the administration of murine antibodies targeting HMW-MAA resulted in a significant reduction of tumor growth (Hafner, Breiteneder et al. 2005). Most of the preclinical studies of HMW-MAA antibodies have utilized murine antibodies, however human scFv's have been generated using phage display technology (Desai, Wang et al. 1998) and, more recently, scFv-Fc fusion molecules were used to construct recombinantly engineered IgG1 antibodies which have demonstrated some anti-tumor activity (Wang, Katayama et al. 2011). However, to-date full-length antibodies with human Fc regions, while having been constructed, have not been assessed for their ability to mediate Fc-mediated immune effector functions in relevant preclinical models. The evaluation of antibodies against HMW-MAA with human Fc regions would be important for the assessment of clinically-relevant antibody candidates for their ability to engage innate immune effector cells as a way of improving their potential clinical utility.

1.2.3 Melanoma Antigens Recognized by the Immune System

Melanoma antigens are instrumental for the detection, treatment, and monitoring of patients with melanoma. Knowledge of antigen expression is crucial to the design of effective immunotherapies. Many melanoma antigens have been identified over the last 20 years by examining T cells and serum antibodies and using SEREX (serological analysis of recombinant DNA expression libraries) platforms (Jager, Stockert et al. 2000; Geuijen, Bijl et al. 2005; Zippelius, Gati et al. 2007). The expression of melanoma antigens has been found to be heterogeneous and identified antigens can broadly be placed in categories such as cancer testis

antigens, differentiation antigens, amplified gene products, and mutation derived proteins (Barrow, Browning et al. 2006). The identification and possible roles of cancer testis antigens, differentiation antigens, and another antigen of interest, the HMW-MAA, will next be discussed.

In melanoma and some other cancers, cancer testis antigens such as the New York esophageal squamous cell carcinoma 1 (NY-ESO-1) antigen and those of the melanoma associated antigen (MAGE) gene family have been found to be overexpressed on cancer cells and have restricted expression on adult testicular germ cells and the placenta and little expression on normal tissues (De Plaen, Traversari et al. 1994; Caballero and Chen 2009). Studies of cancer testis antigen expression have shown the higher expression of such antigens in metastatic melanoma compared to primary lesions (Barrow, Browning et al. 2006). Both humoral and T cell mediated immune responses have been observed against cancer testis antigens such as MAGE-1 and MAGE-3 and NY-ESO-1, with NY-ESO-1 thought to be the most immunogenic antigen based on observations of coordinated antigen-specific CD8+ T cell and antibody responses against this antigen (Caballero and Chen 2009). To-date the functions of these antigens and their role in tumorgenesis are poorly understood.

Differentiation antigens such as gp100, melan-A, tyrosinase proteins (TYRP-1 and TYRP-2) are involved in the pathway of melanin production and are expressed in both melanocytes and melanoma cells. These antigens are strongly expressed in a majority of both primary and metastatic melanomas and are commonly used as markers for the detection of melanoma (Barrow, Browning et al. 2006). The concordant loss of expression of differentiation antigens in melanoma has been found in some individuals to be associated with progressing disease (Trefzer,

Hofmann et al. 2006). However, the mechanisms of immune evasion behind the loss of these antigens are not understood. Notably, it has been found that the treatment with Vemurafenib enhances the expression of these antigens on cells, possibly implicating the MAPK pathway in the expression of these antigens and in immune evasion (Boni, Cogdill et al. 2010).

1.2.3.1 HMW-MAA

One example of an over-expressed protein in melanoma of special interest for this thesis is HMW-MAA, otherwise named chondroitin sulfate proteoglycan 4 (CSPG4), or melanoma chondroitin sulfate proteoglycan (MCSP). The antigen was identified 30 years ago by a monoclonal antibody due to its high expression on melanoma cells and immunogenicity (Wilson, Imai et al. 1981). HMW-MAA is a highly glycosylated transmembrane proteoglycan comprised of a 280 kDa N-linked glycoprotein component (core glycoprotein) and a 450 kDa chondroitin sulfate proteoglycan component expressed on the cell membrane (Figure 1.5). The core protein consists of 2322 amino acids and is comprised of a large extracellular domain, hydrophobic transmembrane region and short cytoplasmic tail (Campoli, Chang et al. 2004; Price, Colvin Wanshura et al. 2011). HMW-MAA has restricted distribution on normal tissues and has been found to be expressed in a high proportion (>80%) of melanomas (Campoli, Chang et al. 2004). The expression of this antigen has also been described in other cancers including triple negative breast cancer, mesotheliomas, squamous cell carcinomas of the head and neck, and glioblastomas, supporting the targeting of this antigen for cancer immunotherapy (Price, Colvin Wanshura et al. 2011; Rivera, Ferrone et al. 2012). Additionally,

HMW-MAA may also hold some importance in the diagnosis of melanoma, having utility in the diagnosis of micrometastases in the sentinel lymph nodes of patients (Goto, Ferrone et al. 2008).

HMW-MAA is thought to play a critical role in linking multiple oncogenic pathways that mediate the progression of malignancy (Price, Colvin Wanshura et al. 2011). HMW-MAA has been described to contribute to the metastatic potential of HMW-MAA expressing cells by modulating or enhancing signaling pathways involved in cell adhesion, proliferation, and invasion (Price, Colvin Wanshura et al. 2011). The antigen is thought to be critical to the growth of melanoma tumors through the modulation of integrin function, heterodimeric receptors important in mediating cell-cell and cell-ECM interaction, and growth factor receptor regulated signaling pathways (Hynes 1992; Price, Colvin Wanshura et al. 2011). HMW-MAA has been shown to activate integrin-regulated focal adhesion kinase (FAK) signaling and receptor tyrosine kinase signaling through the MAPK cascade, signaling cascades which ultimately promote tumor progression (Yang, Price et al. 2004). The antigen has also been implicated in enhancement of α4β1-integrin-mediated melanoma cell spreading along with the regulation of matrix metalloproteinases (MMP)-dependent cell invasion into type I collagen (Iida, Skubitz et al. 1992; Iida, Pei et al. 2001). The mechanisms by which this protein can contribute to metastatic processes are beginning to become more fully understood, as is the efficacy of therapeutic strategies targeting this antigen.

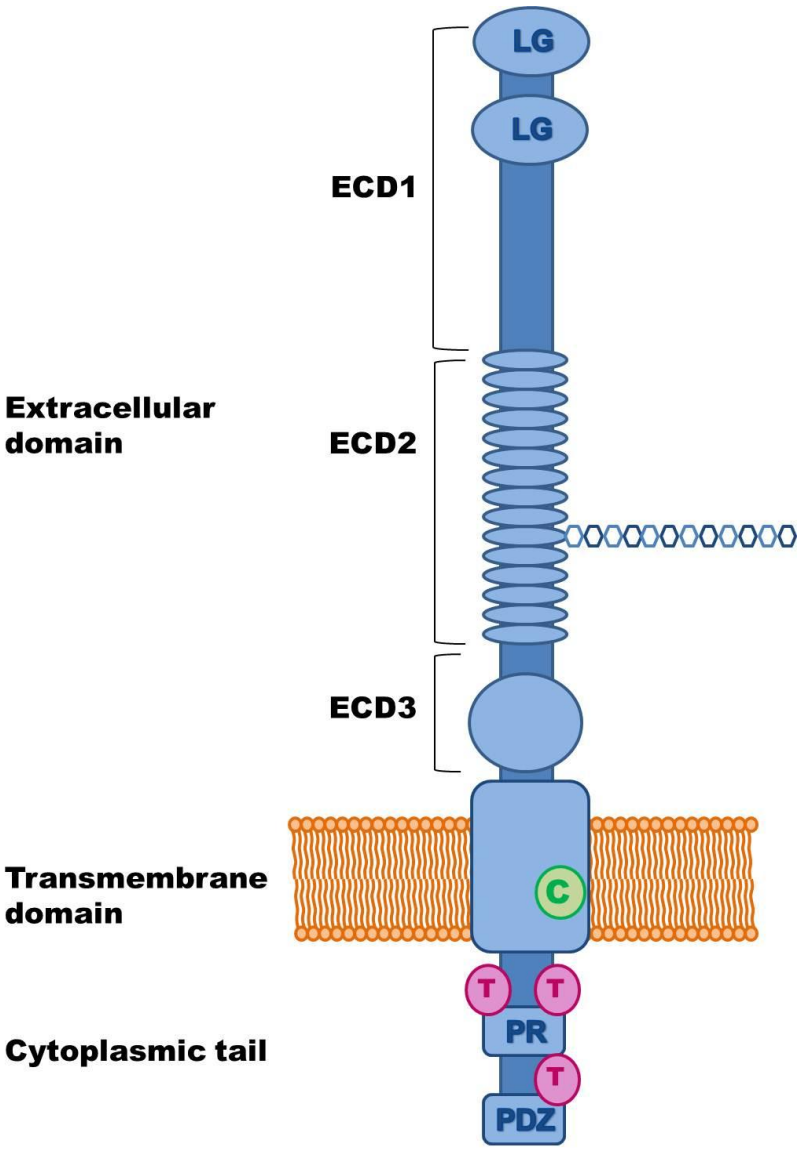


Figure 1.5 Structure of HWM-MAA. Schematic representation of HMW-MAA, a transmembrane proteoglycan comprised of a cytoplasmic C-terminal domain, transmembrane region, and an extracellular domain. The large extracellular domain consists of three subdomains (ECD): (ECD1) globular N-terminal subdomain consisting of laminin G-like regions (LG) and disulfide bonds; (ECD2) 15 chondroitin sulfate proteoglycan repeats known to bind to soluble growth factors and a chondroitin sulfate glycosaminogylcan chain responsible for binding to integrin and MMPs; and (ECD3) the globular subdomain located next the plasma membrane containing lectin binding sites and proteolytic cleavage sites by MMPs. The short cytoplasmic C-terminal domain contains tyrosine residues (T) that serve as phosphoacceptor sites for protein kinase Cα and extracellular signal-regulated kinase (ERK) 1,2 and also a proline-rich region (PR) may facilitate protein-protein interactions. The C-terminus contains a PDZ domain-binding motif (PDZ). Image reproduced from Price, Colvin Wanshura et al. 2011.

There have been a number of preclinical and clinical evaluations of immunotherapeutic strategies aimed at inhibiting the function of HMW-MAA in melanoma. These strategies have included active immunotherapeutic approaches such as the use of peptides mimicking the structure of HMW-MAA epitope (mimotopes or peptide vaccines) (Riemer, Hantusch et al. 2005; Wagner, Hafner et al. 2005; Luo, Ko et al. 2006) or mouse anti-idiotypic antibodies bearing the internal image of HMW-MAA acting as an immunogen for the induction of humoral immunity. This later strategy has been associated with statistically signification prolongation of patient survival in some clinical studies (Mittelman, Chen et al. 1990; Mittelman, Chen et al. 1992). In the preclinical setting, there have been numerous studies of evaluating passive immunotherapeutic approaches using monoclonal antibodies targeting different regions of this protein, and antibodies such as the 225.28S clone have demonstrated direct functions in the blockade of adhesion, migration and anchorage-dependent growth (reviewed in detail in Section 1.2.2). Antibodies targeting HMW-MAA have also been conjugated to immunotoxins and to alpha and beta emitting radioisotopes. In preclinical models, these antibody conjugates have shown cytotoxic effects on melanoma cells (Imai, Nakanishi et al. 1983; Ghose, Ferrone et al. 1991; Liewendahl and Pyrhönen 1993). These studies all highlight the potential of immunotherapeutic strategies to target this melanoma antigen.

Understanding the expression, antigenicity, and functions of melanoma antigens is essential to the diagnosis, prognosis, and treatment of melanoma. Antibodies against melanoma antigens could serve as potential therapeutic agents and also possibly as key diagnostic tools, although no prognostic markers are currently recommended for clinical use in melanoma (Rothberg, Bracken et al. 2009).

Central to the design of effective immunotherapeutic strategies to treat melanoma is the attainment of a better understanding of how the host recognizes and responds to these melanoma antigens. Such an understanding is expected to allow for the more rational selection of antigen targets for both passive and active immunotherapeutic approaches for the treatment of melanoma.

1.3 Immune Responses to Cancer

The immune system plays a dual role in cancer, producing an anti-tumor response on one hand, but also aiding the escape of tumors from eradiation by host immune responses on the other (Dunn, Bruce et al. 2002). A current paradigm in cancer immunology is cancer immunoediting which hypothesizes that there are three phases in the host response to cancer: (1) elimination, (2) equilibrium, and (3) evasion resulting in the change of the host response to cancer from that of immune surveillance to immune escape (Dunn, Bruce et al. 2002; Schreiber, Old et al. 2011). In the initial immune response to cancer, it is thought that innate and adaptive immune systems mount anti-tumor responses to eliminate cancer cells. If not successful, it is thought that cancer cells then remain in a state of equilibrium with the immune system suppressing further malignancy. However, this state is thought to be temporary and eventually leads to immune editing where the continuous pressure on the immune response against tumor cells results in the selection of cells with low immunogenicity leading to the escape of tumor cells from the host anti-tumor immune response (Schreiber, Old et al. 2011). This concept of immunoediting has been supported by clinical observations in

melanoma patients such as the example of the downregulation of NY-ES0-1 expression in relapsed patients following NY-ESO-1 vaccine treatment (Nicholaou, Chen et al. 2011).

Central to immune surveillance is the ability of immune effector cells, such as lymphocytes, to mount a cytotoxic response against tumor cells (Dunn, Bruce et al. 2002). Antigen-specific lymphocyte responses have been described in melanoma with an emphasis on T cells (Lee, Yee et al. 1999; Vence, Palucka et al. 2007), supported by successful clinical case scenarios using immunotherapeutic strategies to enhance T cell anti-tumor responses including dendritic cell vaccines, adoptive T cell therapies, and monoclonal antibodies targeting CTLA-4 (Schadendorf, Ugurel et al. 2006; Dudley, Yang et al. 2008; Kirkwood, Tarhini et al. 2008; Besser, Shapira-Frommer et al. 2010; Hodi, O'Day et al. 2010). There has been much emphasis on the role of T cells in melanoma, while the roles of B cells and their subsets in melanoma and the breadth of humoral immune responses are less understood.

1.3.1 Humoral Responses to Melanoma

It has been reported that B cells constitute a major subset of immune cell infiltrates in primary and metastatic melanoma along with T cells and other immune effector cells such as macrophages (Clemente, Mihm et al. 1996; Erdag, Schaefer et al. 2012); however, the antigen-specific B cell response in the tumor microenvironment is not well understood and insights into antibody responses to melanoma have primarily been inferred from serological studies (Stockert, Jäger et al. 1998; Trefzer, Hofmann et al. 2006).

Reports of tumor-reactive antibodies in the sera of melanoma patients date back over forty years (Lewis, Ikonopisov et al. 1969; Muna, Marcus et al. 1969) and have provided evidence of a humoral response to melanoma. One extensive study performed by Stockert and colleagues examined the presence of antibodies against NY-ESO-1, MAGE-1, MAGE-3, and synovial sarcoma X (SSX2 or HOM-MEL-40) cancer testis antigens along with the differentiation antigens melan-A and tyrosinase proteins in the sera of 127 metastatic melanoma patients (Stockert, Jäger et al. 1998). From this study it was found that twelve patients had reactivity to NY-ESO-1, two patients had reactivity to MAGE-3, and one patient had antibodies reactive to both MAGE-1 and SSX2 and no antibodies to the above differentiation antigens were detected (Stockert, Jäger et al. 1998). These results highlight the presence of humoral immune responses to some cancer testis antigens in a proportion of metastatic melanomas; however, while the study examined a large group of patients, it was restricted to a number of recombinant melanoma antigens. Other groups have examined the reactivity of serum antibodies to identify novel biomarkers for melanoma by screening against autologous cells. One such study led to the identification of bullous pemphigoid antigen 1 in melanoma, which was found in significantly higher amounts in patients with metastatic disease compared to healthy volunteers (Shimbo, Tanemura et al. 2010). These studies all provide evidence of the antigen-specific B cell response to selected melanoma antigens.

Serological studies have provided some meaningful insights into the anti-tumor antibody responses to melanoma; however, the breadth of these responses and changes in the anti-melanoma antibody repertoire of patients with disease progression are not fully understood. Serological studies have been limited to

temporal responses primarily in metastatic patients and do not allow for the evaluation of the full antibody repertoire and assessments of antibody responses occurring in early disease stages. Examining the antibody repertoire of patients' memory B cells could enhance the findings of these serological studies by examining immunologic memory, rather than the temporal responses to melanoma, along with potentially providing insights into the presence and prevalence of an anti-melanoma memory B cell compartment in patients.

Several groups have examined patient peripheral blood B lymphocytes (PBBL) and tumor-infiltrating B lymphocytes (TIL-B) in the quest to discover melanoma-reactive antibodies (Yamaguchi, Furukawa et al. 1987; Kirkwood and Robinson 1990; Yeilding, Gerstner et al. 1992; Punt, Barbuto et al. 1994). From these studies, a few antibodies of the immunoglobulin classes IgM and IgG have been identified from patients PBBL and TIL-B that have reactivity to melanoma cells (Kirkwood and Robinson 1990; Yeilding, Gerstner et al. 1992; Punt, Barbuto et al. 1994). These antibodies have been shown to have reactivity to autologous and allogeneic tumor cells of similar histology and some were found to not react with human fibroblasts (Punt, Barbuto et al. 1994). However, the prevalence of such antibodies and their functional capacity to engage the immune system to mount an anti-tumor response against melanoma cells have not been fully evaluated, likely due to past limitations in the production of antibodies from patient memory B cells (described in Section 1.1.5.2).

Understanding the nature and significance of the anti-melanoma humoral immune response thus warrants further attention and may provide the basis for future strategies aimed at heightening this antibody response in the context of cancer immunotherapy. B cells and other immune cells have been proposed to have dual

roles in cancer: on the one hand they can have anti-tumor effects, but on the other hand, following perturbations in immune tolerance, these cells or subsets of these cells can also contribute to mechanisms of immune escape by tumors.

1.3.2 Mechanisms of Immune Evasion in Tumors

Despite being reported as a highly immunogenic cancer, melanoma is thought to employ multiple mechanisms which can result in the evasion of innate and adaptive immune responses. Metastatic melanoma cells may mediate tumor escape by the secretion of immunosuppressive factors such as the cytokine IL-10, which can inhibit the production of a number of cytokines by different immune cell types potentially resulting in a range of anti-tumor effects such as the impairment of tumor antigen presentation, induction of regulatory T cells (Tregs), and the impairment of macrophage and NK cell functions (Gerlini, Tun-Kyi et al. 2004; Rabinovich, Gabrilovich et al. 2007; Couper, Blount et al. 2008; Emmerich, Mumm et al. 2012). Additionally, tumor cells may secrete pro-angiogenic factors such as VEGF which support tumor growth through the promotion of endothelial cell growth and migration from pre-existing vasculature to the tumor (Claffey, Brown et al. 1996; Hicklin and Ellis 2005). Melanoma cells have also been found to express antibody inhibitory receptors such as FcγRIIB which may function as a decoy, possibly rendering the Fc portion of otherwise cytotoxic antibodies incapable of mounting an immune response against the tumor (Cassard, Cohen-Solal et al. 2008). Additionally, the expression of PD-1 ligand by tumor cells may protect melanoma cells from effector T cell responses in the tumors (Dong, Strome et al. 2002; Iwai, Ishida et al. 2002). These are just a few mechanisms of tumor

induced immune suppression, and many more have been described including those that further impact the functions of immune effector cells, such as NK cells, within the tumor, along with the recruitment of myeloid-derived suppressor cells that can modulate T cell effector functions (Poschke, Mougiakakos et al. 2011; Kerkar and Restifo 2012; Pietra, Manzini et al. 2012).

Leukocytes, such as lymphocytes and macrophages, can be found in high densities in melanoma tumors and have also been implicated in immune suppression which may lead to tumor progression. High numbers of Tregs in patients have been found in the blood and tumor microenvironments, and these cells are thought to be immmuosuppressive and have been correlated with poor prognoses (Nicholaou, Ebert et al. 2009; Wang, Ma et al. 2012). Tregs can also potentially directly suppress B cell responses in secondary lymphoid tissues and inhibit mature antibody responses (Lim, Hillsamer et al. 2005). The upregulation of PD-1 on melanoma antigen-specific CD8+ T cells from TILs compared to normal tissue and peripheral blood leukocytes has been hypothesized to contribute to the impairment of effector antigen-specific effector T cell responses (Ahmadzadeh, Johnson et al. 2009). Additionally, macrophages infiltrating tumors are thought to become polarized to M2 or "alternately activated" macrophages, induced by signals in the tumor microenvironment such as the cytokines IL-4, IL-13, and IL-10 (Mantovani, Sozzani et al. 2002). These alternatively activated macrophages are thought to have altered functions in that they secrete different cytokines and chemokines, have decreased expression of FcγRs, and increased expression of FcεRII (Mantovani, Sozzani et al. 2002). The polarization of macrophages in the tumor can impair the anti-tumor functions of these macrophages by suppressing adaptive immune responses, facilitating matrix remodeling, and promoting

angiogenesis, leading to the promotion of tumor growth and the metastatic processes (Sica, Schioppa et al. 2006).

Some B cell populations have also been described to promote tumor development and metastatic processes. Certain subsets of B cells, such as B-1, have been suggested to contribute to metastatic processes through the expression of glycoproteins involved in cell adhesion, such as the melanoma cell adhesion molecule in mouse models of melanoma (Staquicini, Tandle et al. 2008). The presence of antibodies in premalignant tissue in mouse models has also been suggested to contribute to tumor progression and development through Fc-mediated recruitment and activation of leukocytes which contribute to a chronic inflammatory environment (Tan and Coussens 2007; Andreu, Johansson et al. 2010). Additionally, regulatory B cells (Bregs) have been suggested to be tumor promoting by the secretion of the inflammatory cytokine TNF-α in mouse models of cancer (Schioppa, Moore et al. 2011). The roles of B cells in tumor progression are starting to emerge; most likely the relationship between B cells and tumor progression is complex and variable among B cell subsets. The understanding of the complex nature of B and T cell responses to tumors, especially those of a regulatory nature, can provide insights into novel therapeutic approaches for the treatment of cancer, and the clinical utility of this notion has recently been supported by the success of Ipilimumab.

1.3.3 Th2 Responses in Cancer and Activation of Alternative Humoral Immunity

A dysregulation of immune cells has been noted in metastatic melanoma and many other cancers and a bias towards T helper cell subset 2 (Th2) inflammation has been suggested in both the periphery and local tumor microenvironments (Sheu, Lin et al. 2001; Nevala, Vachon et al. 2009). Th2 cells are a subset of CD4+ T helper cells that promote humoral immunity, producing cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13. Th2 immune responses are also thought to play a role against parasitic infections and are known to be active in the context of immune responses to allergens. These Th2 cytokines are important in regulating humoral immune responses; for example, the cytokines IL-4 and IL-13 stimulate class switching and production of IgE and IgG4 antibodies, with both antibodies being important mediators of allergic immune responses (Gascan, Gauchat et al. 1991; Punnonen, Aversa et al. 1993). IL-10 has also been shown to regulate humoral immune responses by enhancing the production of antibodies of the IgG4 subclass compared to IgE and to direct a "modified Th2 response" which sometimes occurs when IgG4 antibodies are found in the absence of IgE (Jeannin, Lecoanet et al. 1998; Platts-Mills, Vaughan et al. 2001) .

It is hypothesized here that modulation of humoral immunity by local and possibly systemic production of Th2 modulatory cytokines such as IL-4 and IL-10 may occur in metastatic melanoma, which may favor the production of the IgG4 antibody subclass. IgG4 is generally perceived to have weakened effector functions in relation to the other IgG subclasses (Bruggemann, Williams et al. 1987; Aalberse, Stapel et al. 2009). IgG4 is thought to have low immune effector functions due to its low affinity to Fc receptors, poor ability to bind complement,

and its unique ability to interact with other immunoglobulins and engage in Fab arm exchange, leading to antigen bi-specificity of antibodies (van der Zee, van Swieten et al. 1986; van der Neut Kolfschoten, Schuurman et al. 2007; Bruhns, Iannascoli et al. 2009). A polarization of antibody subclasses towards IgG4 production, induced by the Th2 cytokine environment in tumors, could possibly represent a mechanism of impaired humoral immunity through the enhanced production of this antibody subclass, which compared to the other IgG subclasses has relatively weak effector functions.

There has been recent recognition of a group of inflammatory conditions known as IgG4-related diseases such as autoimmune pancreatitis; however, the role of IgG4 in the pathogenesis of these diseases in not clearly understood (Stone, Zen et al. 2012). Furthermore, the association of IgG4 and melanoma has not been described beyond one report dating back 35 years noting alterations in IgG4 proportions in the serum of some malignant melanoma patients (Daveau, Pavie-Fischer et al. 1977). No other associations have thus far been reported in the literature pertaining to alterations of IgG4 levels in sera and other cancers, although more recently one report has described the infiltration of IgG4+ plasma cells in extrahepatic cholangiocarcinoma (Harada, Shimoda et al. 2012). Thus, exploration of the alteration and modulation of antibody subclass in malignancy merits further attention and may represent a mechanism of tumor escape from the host humoral immune response by the preferential production of IgG4 antibodies against TAAs. Studies of the distribution of antibodies of the IgG subclasses in the periphery and metastatic tumor microenvironment could provide further insight into possible Th2 modulations of humoral immune responses in melanoma.

1.3.3.1 Preclinical Evaluations of Tumor-specific Antibodies of the IgE Class

A newly emerging immunotherapeutic approach to harnessing the Th2 responses in cancer is the application of antibodies of the IgE antibody class for passive immunotherapy (Jensen-Jarolim, Achatz et al. 2008; Karagiannis, Josephs et al. 2012). Therapeutic antibodies of the IgE class may have some potential benefits in their clinical application over antibodies of the IgG class and their use in cancer immunotherapy has been supported by a number of preclinical studies (Karagiannis, Josephs et al. 2012). Therapeutic antibodies of the IgE class may confer several advantages over their IgG counterparts in the treatment of solid tumors, including a hundred-fold higher affinity to Fc receptors compared to IgG; lack of IgE inhibitory Fc receptors in tissues and circulation; desirable pharmacokinetic properties such as fast clearance from circulation; the tissue residence of IgE effector cells in tumor lesions such as in melanoma; and the interaction of therapeutic IgE antibodies with FcεRs on macrophages, eosinophils, and mast cells to activate these tissue-resident effector cells against tumors (Karagiannis, Josephs et al. 2012).

Through its Fc region, IgE can recruit and activate Th2 immune effector cells expressing FcεRs resulting in potent inflammatory responses including release of inflammatory mediators and ADCC. It has been hypothesized that Fcε-mediated effector functions could be harnessed against tumor cells using tumor-specific IgE antibodies resulting in potent allergic responses in tumors (Figure 1.6)(Karagiannis, Josephs et al. 2012). Tumor antigen-specific IgE antibodies have been suggested to have anti-tumor functions via the engagement of FcεRs on monocytes/macrophages in a number of *in vitro* and *in vivo* investigations (Karagiannis, Wang et al. 2003; Karagiannis, Bracher et al. 2007; Karagiannis,

Bracher et al. 2008; Karagiannis, Singer et al. 2009). A tumor-specific IgE therapeutic holds particular interest in melanoma with reports of high densities of FcεR bearing immune cells in the solid tumor, such as macrophages and mast cells (Duncan, Richards et al. 1998; Tth, Tth-Jakatics et al. 2000; Erdag, Schaefer et al. 2012). Thus, the immunotherapeutic use of the IgE antibody class may yield a new class of promising agents with potential to mount a specific and potent anti-tumor immune response within the context of Th2-driven immune responses already present in cancers such as melanoma, and the concept of tumor-specific IgE therapy awaits further examination in a clinical setting.

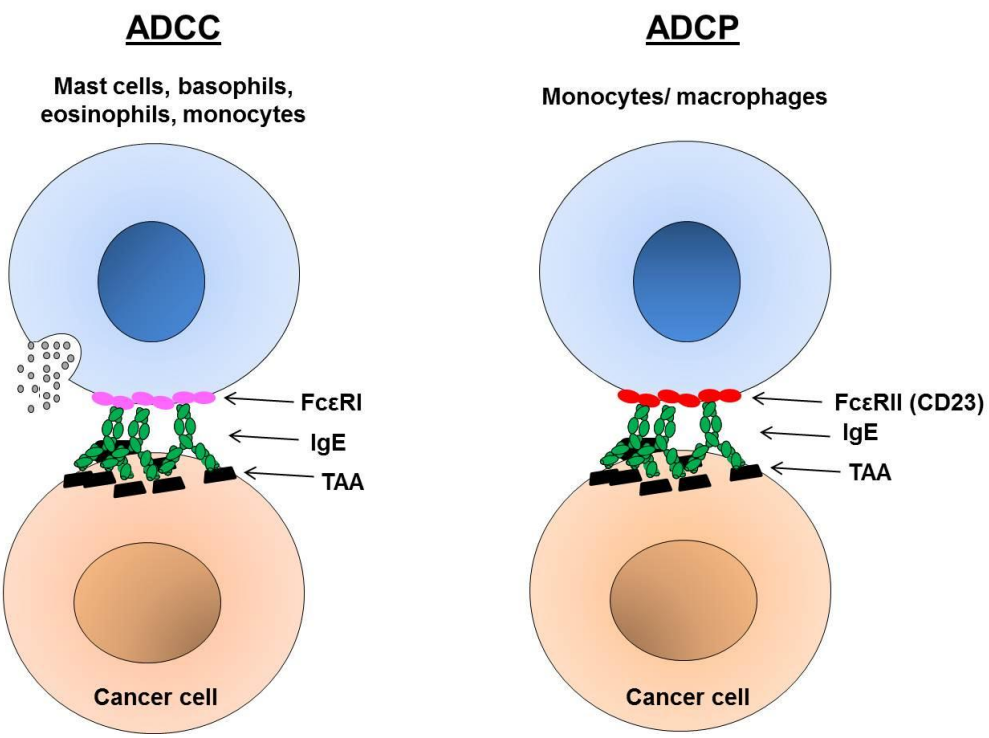


Figure 1.6 Mediation of ADCC or ADCP by IgE antibodies targeting tumor associated antigens. Antibodies of the IgE class targeting TAAs can mediate cellular mechanisms of action resulting in the destruction of tumor cells through the interaction with FcεRs present on immune effector cells. IgE, bound to TAAs on cancer cells in a multimeric fashion can interact with the FcεRI expressed on immune cells such as mast cells, basophils, eosinophils and monocytes resulting in multimerization, crosslinking and activation resulting in the release of inflammatory mediators such as leukotrines and histamines. IgE, bound to TAAs on cancer cells in a multimeric fashion can also interact with the FcεRII (CD23) to mediate the phagocytosis of tumor cells coated with IgE antibodies (Karagiannis, Bracher et al. 2007).

Preclinical studies have demonstrated the use of the IgE antibody class against TAAs to harness an allergic response against cancer cells to mediate tumor cell cytotoxicity. Engineered IgG and IgE antibodies (MOv18 IgG and IgE) directed at FRα were both observed to inhibit tumor cell growth in a mouse severe combined immunodeficiency (SCID) xenograft model of ovarian carcinoma in the presence of human immune effector cells (Gould, Mackay et al. 1999). Following these findings, later studies revealed the important role of monocytic cells as effector cells in these models and the ability of a tumor-specific IgE antibody to mediate ADCC through FcεRs on monocytes and other FcεR bearing immune cells such as eosinophils (Karagiannis, Wang et al. 2003; Karagiannis, Bracher et al. 2007; Karagiannis, Bracher et al. 2008). Further efforts to evaluate tumor-specific IgE antibodies resulted in the construction of an IgE homologue of Trastuzumab. This antibody was also shown to mediate ADCC in the presence of breast cancer cells and monocytes and to trigger the degranulation of mast cells following antibody crosslinking (Karagiannis, Singer et al. 2009). Interestingly, these studies revealed the different functions of Trastuzumab IgE, which was found to primarily mediate ADCC, compared to Trastuzumab (IgG1) which was found to primarily mediate ADCP in functional flow cytometry assays designed to simultaneously measure ADCC and ADCP (Karagiannis, Singer et al. 2009). Others have also tested the efficacy of tumor-specific IgE antibodies in mouse models of breast and ovarian cancer, demonstrating the prolonged survival or restriction of tumor growth in transgenic FcεRI mice following the administration IgE antibodies targeting Her2/neu and mucin 1 (Daniels, Leuchter et al. 2012; Teo, Utz et al. 2012). In summary, these preclinical data support the efficacy of antibodies of the IgE class to mediate cytotoxicity against cancer cells through the engagement and activation

of FcεRs and present an alternative strategy to IgG-based immunotherapeutic approaches.

IgE is implicated in mediating allergic reactions and the administration of a tumor-specific IgE antibody in patients comes with the primary concern of the possible induction of undesirable allergic responses. While a tumor-specific IgE antibody could mediate its therapeutic effect through the crosslinking of IgE molecules upon binding to tumor cell surface antigens and FcεRI expressing cells, such crosslinking would be undesirable in the blood because, in certain circumstances, it could lead to possible type 1 allergic hypersensitivity which could range from a mild irritation to anaphylactic shock. Such concerns have been explored using human *ex vivo* allergy models which showed that the induction of possible type 1 hypersensitivity in blood required large concentrations of circulating soluble antigens or tumor cells, concentrations not normally found in patients with metastatic disease (Rudman, Josephs et al. 2011). Using these *ex vivo* allergy models, Rudman and colleagues also demonstrated a lack of basophil activation in the blood of patients with ovarian cancer following incubation with a tumor-specific IgE antibody, even in the presence of soluble antigen and in the presence of autoantibodies to this antigen (Rudman, Josephs et al. 2011). These *ex vivo* findings suggest that there may be a relatively low risk of type 1 hypersensitivity following the systemic administration of IgE to patients, even in the presence of a monomeric soluble tumor antigen and circulating tumor cells in levels greater than physiologically found in patients. Importantly, the Fc-mediated mechanism of action of IgE antibodies depends on the crosslinking of antibodies, and it is desirable that such crosslinking occurs only in solid tumors in the presence of multivalent antigens presented on tumor cells.

The preclinical efficacy of IgE antibodies targeting tumor antigens in breast and ovarian carcinomas taken together with the infiltration of IgE effector cells such as macrophages and mast cells in melanoma tumors support the design and development of IgE immunotherapies against melanoma cell surface antigens. The engineering of IgE antibodies targeting cell surface melanoma antigens such as HMW-MAA may combine the direct Fab-mediated functions of an antibody, which could be to block melanoma cell adhesion and invasion along with the potent Fc-mediated mechanisms of action of IgE, with the aim of harnessing the Th2 responses in melanoma tumors.

1.4 Hypotheses and Aims

Given the importance of understanding the complexity of the immune response to melanoma in the design of effective immunotherapies, this thesis aims to firstly characterize key elements of the humoral immune responses to melanoma. This entails the characterization of the antibody response to melanoma residing in the periphery along with the local tumor environment. Additionally, the function of antibodies directed at melanoma cells, including antibodies of the IgE class, are evaluated using *in vitro* models to examine potentially novel immunotherapies that specifically target melanoma cells.

The first part of this thesis investigates an approach to discover antibodies from cancer patients through the application of techniques to activate B cells to secrete high levels of antibodies (Traggiai, Becker et al. 2004), a technique which has yet

to be applied to the cancer field. Central to this is the establishment of *ex vivo* antibody-secreting B cell cultures from patients and healthy volunteers, and the design and development of screening tools to identify tumor-specific antibodies from these B cell culture supernatants (Chapter 3).

The second part of this thesis examines humoral immune responses in melanoma (Chapter 4) by characterizing the anti-melanoma antibody compartment from peripheral blood B cells from a patient cohort, utilizing the methods designed in Chapter 3. These studies provide insight into the extent of the humoral immune response to melanoma cells existing in patient circulating memory B cells. This work expands upon previous serological studies by assessing the reactivity of antibodies from the human memory B cell antibody repertoire, characterizing long term antibody responses to melanoma. While a decrease in the memory B cell phenotype has been more recently described in patients with Stage IV melanoma (Carpenter, Mick et al. 2009), any changes in the anti-melanoma antibody compartment have not been characterized with progressing disease, and it is hypothesized here that, following the immunoediting paradigm, the humoral immune responses to melanoma are modulated with disease progression.

Additionally, modulations of antibody responses to melanoma are evaluated from a unique perspective. Immunosuppressive cytokines such as IL-10 have been widely described in the melanoma tumor microenvironment, and the presence of IL-10 has been demonstrated to increase the production of IgG4 (Jeannin, Lecoanet et al. 1998; Satoguina, Weyand et al. 2005). Taken together, it was investigated whether IL-10 or other Th2 cytokines could impair humoral immunity in the periphery and tumor microenvironment by the polarization of antibody subclass production towards the weakened IgG4 subclass (Chapter 4).

The last part of this thesis evaluates the function of antibodies targeting melanoma cell surface antigens (Chapter 5). First, a novel monoclonal melanoma-specific antibody derived from a patient is characterized, and the ability of this patient-derived antibody to engage immune effector cells is evaluated using *in vitro* models. Next, engineered antibodies of the IgG and IgE class targeting HMW-MAA are evaluated using *in vitro* models for their ability to restrict the adhesion and invasion of melanoma cells. Additionally, the immune effector functions of these HMW-MAA antibodies are evaluated in assays examining ADCC and ADCP using primary monocytes, since it is hypothesized that different antibody classes can each have potent but different Fc effector mechanisms against tumor cells. Comparing the function of a melanoma-specific IgE antibody to its IgG counterpart may provide further support for future clinical application of IgE, a novel class of antibody for the treatment of melanoma.

Chapter 2: Materials and Methods[2]

[2] Figure 2.1 and Sections 2.7.5, 2.8, 2.10.3, and 2.11.1-2 are reproduced from Gilbert, Karagiannis et al. 2011.

2.1 Human Subjects

After obtaining informed consent, peripheral blood was isolated from healthy volunteers and from patients with melanoma. Patient melanoma tumor specimens were also provided following informed consent from patients prior to the surgical removal of tumor. Patients were staged and classified according to the American Joint Committee on Cancer Melanoma Staging and Classification criteria (Balch, Gershenwald et al. 2009). Human studies were all conducted in accordance with the Helsinki Declaration and approved by the Guy's Research Ethics Committee, St Thomas' Hospital, London UK, Ethics number 08/H0804/139.

2.2 Tissue Culture

2.2.1 General Reagents

The tables following list the source of cell lines (Table 2.1) and the reagents used to culture these cells (Table 2.2).

Table 2.1 Mammalian Cell Lines

Cell line	Type of cell line	Supplier	Code number
A-2058	Metastatic human melanoma derived from a lymph node	ATCC	CRL-11147
A-375	Metastatic human melanoma derived from skin	ATCC	CRL-1619
B95-8	Marmoset cell line infected with human EBV	ATCC	VR-1492
Fibroblasts	Human skin fibroblasts derived from the dermis	C. Hundhausen, King's College London (KCL)	Gift
G-361	Metastatic human melanoma derived from skin	ATCC	CRL-1424
Melanocytes	Human epidermal melanocytes derived from neonatal foreskin	ATCC	200-012
SK-BR-3	Metastatic human adenocarcinoma derived from mammary gland	ATCC	HTB-30
SK-MEL-2	Metastatic human melanoma derived from skin	ATCC	HTB-68
SK-MEL-28	Metastatic human melanoma derived from skin	ATCC	HTB-72
U-937	Human monocytic-like cells derived from non-Hodgkin lymphoma	ATCC	CRL-1593.2
WM-115	Human primary melanoma derived from skin	ATCC	CRL1675

Table 2.2 Tissue Culture Reagents

Name	Supplier	Code number
Dermal Cell Basal Medium	ATCC	PCS-200-030
Dimethyl sulfoxide (DMSO)	Sigma	472301
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco	41966-029
Eagle's Minimum Essential Medium (MEM) with Earle's Salts	PAA	E15-825
Fetal calf serum (FCS)	Gibco	10106-169
Low Serum Growth Supplement	Invitrogen	S-003-10
McCoy's 5a Medium Modified	Invitrogen	36600021
Medium 106	Invitrogen	M106500
Melanocyte Growth Kit	ATCC	PCS-200-041
Phosphate buffered saline 1x pH 7.2 (PBS), sterile	Gibco	20012
Penicillin (100 U/mL) and streptomycin (100 µg/mL) (PenStrep)	PAA	P11-010
RPMI 1640	Gibco	21875-0
StemPro® Accutase® Cell Disassociation solution	Gibco	A1110501
Trypsin-ethylenediaminetetraacetic (EDTA) (0.05%)	Gibco	25300-054

2.2.2 Cell Line Maintenance

Tissue culture was performed under sterile conditions in a Nuaire™ laminar flow hood using aseptic technique. Cells were grown in a humidified atmosphere at 37˚C in 5% CO_2 in the Nuaire™ CO_2 Air-Jacketed Incubator. Cell culture medium was supplemented with 10% FCS and 1% PenStrep, referred to as standard additives unless otherwise specified. Cell lines were maintained for a maximum of 30 passages. Adherent cell lines were passaged once they reached 80-90% confluence. To passage adherent cells, medium was removed and the cell monolayer was washed with PBS and then a minimal amount of 0.05% Trypsin-EDTA was used to cover the monolayer. Cells were then placed in incubator for the minimal time required for cell detachment (typically 2-10 minutes) which was

monitored by light microscopy. Detached cells were promptly placed in excess of the culture medium they were grown in and centrifuged (Hettich Rotina 420R Centrifuge, used for all experiments unless otherwise specified) at 200 x g for 5 minutes at room temperature. Cell supernatants were then discarded and cells were split at an optimal ratio and placed in a new tissue culture flask. Culture medium for non-adherent cell lines was replaced by centrifugation of cell culture flask contents for 5 minutes at 200 x g at room temperature. Supernatant was then discarded and replaced with fresh medium. Non-adherent cell lines were passaged once cell concentrations were above recommended cell density. Cells were stored in liquid nitrogen in 1mL aliquots of 10 x 10^6 cells in 0.9 mL of freezing media (90% FCS and 10% cell-specific culture media) plus 0.1 mL of DMSO (final concentration 10%). Prior to being placed in liquid nitrogen, cells were placed in a cryopreservation freezing container in a -80˚C freezer where the cells were cooled at a rate of -1˚C/minute.

The specific maintenance for each cell line used in the experiments described herein is listed below.

A-2058 Cell Line: The adherent human malignant melanoma cell line A-2058 was used for *in vitro* screening assays. A-2058 cells were maintained in DMEM with standard additives and medium was replaced three times per week. Cells were passaged as detailed above and split at a ratio of 1:6 to 1:12.

A-375 Cell Line: The adherent human malignant melanoma cell line A-375 was used for *in vitro* screening assays along with studies of cell migration, adhesion, cytotoxicity, ADCP and ADCC. A-375 cells were maintained in DMEM with

standard additives and medium was replaced three times per week. Cells were passaged as detailed above and split at a ratio of 1:3 to 1:8.

B95-8 Cell Line: The EBV infected marmoset lymphoblastoid cell line B95-8 was used as a source of EBV (Miller and Lipman 1973). A detailed description of the methodology used to produce EBV from these cells is described in Section 2.2.3. B95-8 cells were maintained in RPMI 1640 medium at a density of 1-2 x 10^6 cells per mL.

Fibroblast: Human dermal fibroblasts isolated from a healthy volunteer were a gift from Dr. Christian Hundhausen, KCL. Primary fibroblasts were isolated from adult human skin by separating the dermis from the epidermis after an overnight incubation with Dispase (1 mg/mL concentration) at 4°C. The dermis was then cut into 1 mm^2 pieces using a scalpel. The dermis was next incubated in 0.1% Collagenase I diluted in RPMI 1640 medium and then placed in a 50 mL conical tube at 37°C on a shaker for a minimum of 2 hours with intermittent mixing. Next, the solution was filtered using a cell strainer with a 100 µm pore size, the filtrate was washed two times with PBS, resuspended in Medium 106 and supplemented with Low Serum Growth Supplement. Cells were maintained using the above media and were split once 80-90% confluence was reached.

G-361 Cell Line: The adherent human malignant melanoma cell line G-361 was used for *in vitro* screening assays. G-361 cells were maintained in McCoy's 5a Medium modified with standard additives and medium was replaced three times per week. Cells were passaged as detailed above and split at a ratio of 1:2 to 1:4.

Melanocytes: Primary neonatal human epidermal melanocytes were used for *in vitro* screening assays as a control. Cells were grown in Dermal Cell Basal Medium with the addition of a Melanocyte Growth Kit containing L-glutamine, recombinant human insulin, ascorbic acid, epinephrine, calcium chloride, and a proprietary supplement containing FCS and other factors. Medium was replaced twice per week and cells were split a ratio of 1:2 to 1:4.

SK-BR-3 Cell Line: The adherent human breast cancer cell line SK-BR-3 was used for the development of an *in vitro* screening assay. SK-BR-3 cells were maintained in DMEM with standard additives. Medium was replenished three times per week and cells were split at a ratio of 1:2.

SK-MEL-2 Cell Line: The adherent human malignant melanoma cell line SK-MEL-2 was used for *in vitro* screening assays. SK-MEL-2 cells were maintained in MEM with standard additives and medium was replenished three times per week. Cells were split at a ratio to 1:3 to 1:6.

SK-MEL-28 Cell Line: The adherent human malignant melanoma cell line SK-MEL-28 was used in *in vitro* screening assays. SK-MEL-28 cells were maintained in MEM with standard additives and medium was replenished three times per week. Cells were split at a ratio to 1:3 to 1:6.

U-937 Cell Line: The human monocyte-like cell line U-937 was used to establish and perform *in vitro* screening assays and as effector cells in some cytotoxicity assays. U-937 cells were maintained in RPMI media with standard additives. A cell density of 2 $x10^5$ to 2 $x10^6$ cells per mL was maintained and the medium was replaced every 3 to 4 days.

WM-115 Cell line: The adherent human melanoma cell line WM-115 was used for *in vitro* screening assays. WM-115 cells were maintained in MEM with standard additives. Media was replenished three times a week and split at a ratio to 1:3 to 1:6.

2.2.3 Preparation of EBV from B95.8 Cells

This section documents the methods used to produce EBV, a double-stranded DNA virus belonging to the herpes virus family, for B cell immortalization and transformation experiments. EBV can persist in the nuclei of infected B cells resulting in the lytic replication of virons and *in vitro* it has been found to transform B cells into proliferating lymphoblastoid cell lines by the secretion of EBV-encoded latent proteins and RNA (Kuppers 2003). The EBV producing lymphoblastoid marmoset cell line B95-8 sheds EBV (of human origin) into cell culture supernatants (Miller and Lipman 1973).

B95-8 cells were grown until confluent and then starved of nutrients for 7-10 days. Next the contents of the tissue culture flask, including cells and supernatant, were frozen at -20˚C overnight. The virus was then harvested from the starved cells by first centrifuging the thawed contents of the flask for ten minutes at 400 x g. Next, the supernatant was removed and centrifuged again for another ten minutes at 700 x g. After centrifugation, the supernatant was removed, filtered using a 0.45 µm filter and stored at -20˚C until used.

2.3 Primary Cell Isolation

2.3.1 General Reagents

The reagents used to isolate primary human cells are listed in the table below.

Table 2.3 Reagents Used for Primary Cell Isolation

Name	Supplier	Code number
EBV	--	Refer to Section 2.2.3
CpG ODN	Operon	Custom order
EDTA 0.5 M pH 8.0, ultrapure	Gibco	15575-020
FCS	Gibco	10106-159
Filcoll-Paque PLUS™	GE Healthcare	17-1440-03
GentleMACS C tubes	Miltyeni Biotec	130-093-237
PenStrep	PAA	P11-010
PBS, sterile	Gibco	20012
RosetteSep™ Human B Cell Enrichment Cocktail	Stem Cell Technologies	15024
RosetteSep™ Human Monocyte Enrichment Cocktail	Stem Cell Technologies	15068

2.3.2 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were isolated from blood by density gradient centrifugation. Equal volumes of blood and PBS + 2% FCS were gently mixed at a maximum volume of 15 mL and slowly pipetted on top of 15 mL of Ficoll-Paque PLUS™ density medium in a 50 mL conical vial. The tube was centrifuged for 20 minutes at 1200 x g with no brake at room temperature. After centrifugation, PBMCs were removed from the density medium and plasma interface.

2.3.3 Isolation of B cells from Peripheral Blood

B cells were isolated from whole blood by performing negative selection using the RosetteSep® B cell enrichment cocktail containing monoclonal antibodies to the following human cell surface antigens: Glycophorin A, CD2, CD3, CD16, CD36, CD56, and CD66b. For each blood sample, 50µL of RosetteSep® B cell enrichment cocktail was added to 1 mL of blood and this solution was mixed gently and incubated for 20 minutes at room temperature. The sample was next diluted in an equal volume of PBS + 2% FCS and mixed gently. The diluted sample, at a maximum volume of 15 mL, was slowly pipetted on top of 15 mL of Ficoll-Paque PLUS™ density medium in a 50 mL conical vial so that any mixing was minimized. The tube was centrifuged for 20 minutes at 1200 x g at room temperature with no brake. After centrifugation, enriched cells were removed from the density medium and plasma interface.

2.3.4 Isolation of Monocytes from Peripheral Blood

Human monocytes were isolated by negative selection using the RosetteSep® monocyte cell enrichment cocktail containing tetrameric antibody complexes to the following human cell surface antigens: Glycophorin A, CD2, CD3, CD8, CD19, CD56, and CD66b. Monocytes were isolated in the same manner as B cells as previously described (Section 2.3.3) with the addition of 1 mM EDTA to the 2% FCS/PBS buffer.

2.3.5 Isolation of Cells from Tumor Specimens

Melanoma tumor specimens from skin or lymph nodes were processed on the same day as surgery. Specimens were rinsed with PBS/2% PenStrep, cut into approximately 2 mm^2 pieces, and placed into RPMI 1640 media containing 1% PenStrep, 10% FCS, 30% EBV supernatant, and 2.5 ng/mL of the TLR9 agonist CpG ODN in a Nuaire™ CO_2 Air-Jacketed Incubator set at 37˚C and 5% CO_2 overnight. The following day, the contents were strained over a filter with a pore size of 100 µm. After filtering, the collected liquid was centrifuged at 300 x g for 8 minutes at room temperature and the cell pellet was resuspended in RPMI 1640 media and returned to 37˚C incubator. The tissue remaining on the filter was then removed and dissociated using the gentleMACS™ dissociator (Miltyeni Biotec) using RPMI 1640 media/10 % FCS, gentleMACS™ C tubes and the "m imp tumor 01.01" gentleMACS™ program. The contents of the tube were filtered using a 100 µm filter and transferred to a 50 mL conical vial, centrifuged for 8 minutes at 300 g and the cell pellet was resuspended in RPMI 1640 media and added to cells collected at earlier steps.

2.4 Human Primary B cell Cultures

2.4.1 General Reagents

The culture of human B cells was performed using the materials and reagents listed below.

Table 2.4 General Reagents and Materials Used for Establishing B cell Cultures

Name	Supplier	Code number
CpG ODN	Operon	Special order
EBV	--	See Section 2.2.3
Tissue culture 96 well round bottom Plates	Nunc	163320
RPMI 1640 Medium	Gibco	21875-0

2.4.2 Peripheral Blood B cell Cultures

Immediately after isolation from blood, B cells were added onto 96 well round bottom plates at densities of 100 to 1000 cells per well. Each culture well contained 40 µL of RPMI media, 60 µL of EBV supernatant and 2.5 ng/mL of the TLR 9 agonist CpG ODN. Using the Gammacell 10000 Elite vs. 2.09 cell irradiator, autologous PBMCs were irradiated at a dose of 3010 cGy. Irradiated PBMCs at a concentration of 3×10^5 cells in 100 µL of RPMI 1640 medium were added to the B cell cultures as feeder cells. Thus B cells were cultured in media containing 2.5 ng/mL CpG ODN/30% EBV supernatant/70% RPMI 1640.

2.4.3 Tumor-derived B cell Cultures

Mixed populations of cells isolated from tumors (see Section 2.3.5) were plated in 96 well round bottom plates at approximate densities of 5 x10^5- 5 x10^6 cells per well in RPMI 1640/30% EBV supernatant/2.5 ng/mL CpG ODN. Cells were maintained in the Nuaire™ CO_2 Air-Jacketed Incubator set at 37˚C and 5% CO_2.

2.5 Immunocytochemical Methods

2.5.1 General Reagents

The specific binding of antibodies to cells was qualitatively detected using ICC methods with the reagents listed below.

Table 2.5 General Reagents Used for Immunocytochemical Analyses

Name, chemical formula, formula weight	Supplier	Code number
Antibody Diluent	Dako	S3022
Acetone, CH3COCH3, 58.08	Sigma	320110
3,3' Diaminobenzidine (DAB) chromogenic substrate for horseradish peroxidase (HRP)	Dako	K3466
Ethanol	BDH Prolabo	20821.330
Formaldehyde solution, CH_2O, 30.03	Sigma	F1268
Human AB serum	Sigma	H4522
1 M Hydrochloric acid (HCl), HCl, 36.46	Sigma	H1758
Mayer's Haematoxylin	Merck	1.09249
Neutral mounting medium (DPX)	BDH	360294H
Tris buffered saline, 25mM Tris, 0.15M NaCl, pH 7.2 (TBS)	Sigma	28379

2.5.2 Antibodies

The specific binding of antibodies to cells was qualitatively detected using ICC methods with the antibodies listed below.

Table 2.6 Antibodies Used for Immunocytochemical Analyses

Specificity/ Clone	Species specificity	Conjugate	Host species	Supplier	Code number
HMW-MAA /LHM2	Human	-	Mouse	Invitrogen	41-2000
IgG	Mouse	Peroxidase	Rabbit	Dako	P0268

2.5.3 Preparation of Cytospins

Cytospins for ICC analyses were prepared by washing cells of interest in PBS/10% FCS. Cells were then diluted to a concentration of $2x10^5$ cells in 100 µL PBS and added to a microscope slide. Using the Shandon Cytospin® 4 Cytocentrifuge (Thermo Scientific) cells were spun for 5 minutes at approximately 50 x g, then removed from the centrifuge and left to dry overnight. On the following day, cells were fixed with cold (-20˚C) acetone for 10 minutes and left to air dry for 30 minutes. Cytospins were either used immediately or placed in a slide box at -80˚C covered in foil until day of use.

2.5.4 Detection of Tumor-specific Antibodies by Immunocytochemistry

For ICC analyses, cells on cytospins were circled using a hydrophobic barrier pen. Next, 250 µL of antibody diluted in antibody diluent was added per cytospin and incubated overnight at 4˚C. Cytospins were then washed in TBS three times with a

5 minute incubation of wash buffer between washing steps. Secondary antibody labeled with HRP was diluted 1:100 and 250 µL was added to each cytospin and incubated for 45 minutes at room temperature. Cytospins were then washed as described above. Next DAB chromogenic substrate for HRP was added to each cytospin. Color was allowed to develop for 1 to 5 minutes and the reaction was stopped by washing with TBS as previously described. Slides were washed in tap water and cells were counterstained by immersion in Mayer's Haematoxylin for approximately 1 minute. Slides were washed again in tap water and excess stain was removed by briefly dipping the slides into 0.5%HCl/70% ethanol, and then slides were washed once more in tap water until the nuclei were blue. Slides were dehydrated in a series of graded ethanol concentrations (50, 70, 90 and 100%) for 3 second intervals each and then placed in xylene for 1 minute. Slides were then mounted with glass cover slips using DPX.

2.6 Flow Cytometry

The detection of cell surface or intracellular protein expression was performed using flow cytometry. Using fluorescently labeled antibodies, the expression of desired antigens could be detected on cells of interest. Flow cytometry experiments were conducted with either a FACSAria or a FACSCanto (BD Biosciences) instrument operated with FACSDIVA™ software (BD Biosciences) and data were analyzed using Flow Jo (Tree Star, Ashland, OR) software unless otherwise specified. The general reagents (Table 2.7) and antibodies (Table 2.8) used in flow cytometric assays are listed below.

2.6.1 General Reagents

Flow cytometric analyses were conducted using the reagents listed below.

Table 2.7 General Reagents Used for Flow Cytometry

Name	Supplier	Code number
BD Cytofix™	BD Biosciences	55465
Normal goat serum (NGS)	Sigma	G9023
IntraStain Fixation and Permeabilization Kit for Flow Cytometry	DAKO	K2311
PBS, sterile	Gibco	20012

2.6.2 Antibodies

Flow cytometric analyses were performed using the antibodies listed below.

Table 2.8 Antibodies Used for Flow Cytometry

Specificity	Clone	Conjugate	Host species	Supplier	Code number
CD22	S-HCL-1	Allophycoyanin (APC)	Mouse	BD Biosciences	333145
CD27	M-T271	Fluorescein Isothiocynanate (FITC)	Mouse	BD Biosciences	555440
CD89	A59	Phycoerthrin (PE)	Mouse	BD Biosciences	555686
Her2/neu		--	Human	Genentech	Herceptin™
HMW-MAA	LHM2	--	Mouse	Invitrogen	41-2000
HMW-MAA IgG and IgE Classes	225.28S	--	Human	KCL	Gift
IgA + IgG + IgM*	--	FITC	Goat	Jackson Immuno Research	109-096-64
IgG heavy + light Chain*	--	FITC	Goat	Jackson Immuno Research	109-096-064
IgG Fcγ	--	FITC	Goat	Jackson Immuno Research	109-096-098

* $F(ab')_2$ fragments

2.6.3 Cell Surface Staining

Antibodies bound to cell surface antigens were also detected on live cells by flow cytometry. Adherent cells were detached using StemPro® Accutase® cell dissociation solution. For the detection of tumor specific antibodies, live tumor cells were incubated with isotype control antibody or cell culture supernatants containing antibodies secreted by *ex vivo* activated B cells for 30 minutes at 4˚C. Cells were then washed with fluorescence-activated cell sorting (FACS) buffer, PBS/2% NGS, and incubated with fluorescently labeled secondary antibodies against the human Ig Fc portion of the antibody for 30 minutes at 4˚C. Expression of cell surface antigens on melanoma cell lines was evaluated as described above using antibodies recognizing known cell surfaces antigens such as the HMW-MAA.

2.6.3.1 Phenotypic Analysis of Memory B cell Compartments

Phenotypic analyses of the memory B cell compartments from healthy volunteer blood and also from the blood of patients with Stage I-IV melanoma were performed with antibodies which stained for a mature B cells (CD22) and memory B cell (CD27) markers (Klein, Rajewsky et al. 1998; Sato, Tuscano et al. 1998). B cells were freshly isolated using the Rosette Sep® B cell enrichment cocktail, suspended in FACS buffer, and then incubated with fluorescently labeled antibodies to CD22 and CD27 for 30 minutes at 4˚C. Cells were washed as described above and immediately analyzed on the flow cytometer.

The portion of memory B cells was estimated with the following formula:

$$\%\ Memory\ B\ cells = \frac{\%\ CD\ 27\ and\ CD22\ double\ positive\ cells}{\%\ CD22\ positive\ cells}$$

All analyses were performed by calculating the percentage of CD22 positive and also CD22 and CD27 double positive cells (CD22+CD27+) relative to isotype antibody controls (Figure 2.1).

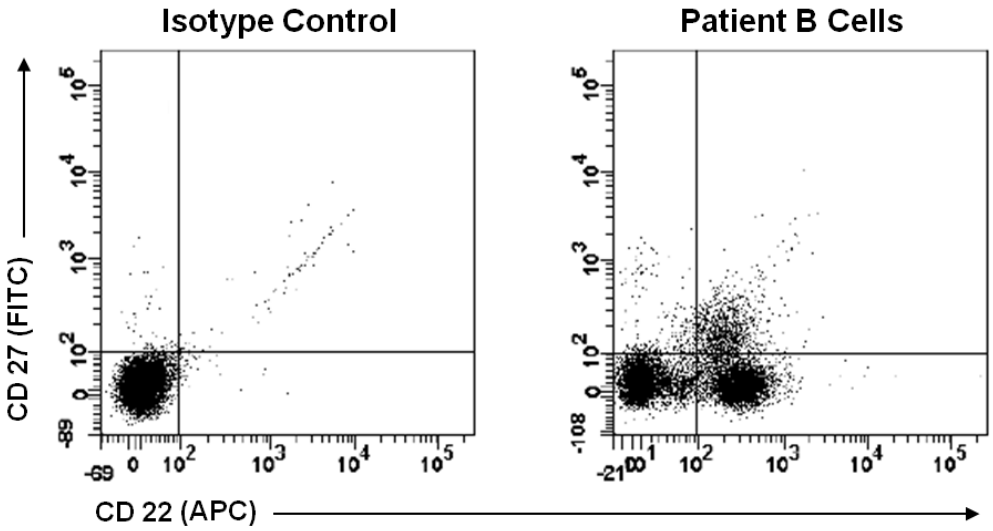


Figure 2.1 Phenotypic analysis of memory B cell populations from human peripheral blood cells. Freshly isolated peripheral blood B cells were stained with anti-human CD27 and CD22 antibodies and memory B cell subsets were evaluated. Gates were set using non-specific anti-murine CD22 and CD27 antibodies as isotype controls. Dot plots were derived from a representative patient with melanoma (M144).

2.6.4 Intracellular Staining

For the intracellular staining of human primary B cells, B cells were isolated from the peripheral blood of patients (n=3), cultured *ex vivo* for 18 days, removed from culture wells (n=360), pooled, and washed in appropriate culture media. Staining was performed using the IntraStain Fixation and Permeabilization Kit according to the manufacturer's instructions. B cells were first fixed for 15 minutes, washed in buffer, and centrifuged for 5 minutes at 500 x g at room temperature. After removal of the wash supernatant from tubes, cells were permeabilized, the desired fluorescently labeled antibody was added to tubes, and cells were incubated in the dark for 15 minutes. B cells were then washed in buffer as described previously, resuspended in buffer and analyzed immediately analyzed using a flow cytometer.

2.7 ELISA

Measurement of immunoglobulin concentration and reactivity to antigens on the surface of cells was performed by ELISA. The general reagents (Table 2.9) and antibodies (Table 2.10) used for ELISAs are listed below.

2.7.1 General Reagents

Table 2.9 General Reagents Used for ELISA

Name	Supplier	Code number
BupH™ carbonate-bicarbonate Buffer, 0.2M pH 9.4	Pierce	0028382
Formaldehyde solution	Sigma	F1268
Hank's Buffered Salt Solution (HBSS), calcium and magnesium Free	Sigma	14170-088
1 M HCl	Sigma	H1758
Marvel dried skim milk	Premier International Foods (UK) Ltd	--
Maxisorp™ 96 well plates	Nunc	442404
σ-Phenylenediamine dihydrochloride (OPD) Tablet	Sigma	P6912
PBS 1x pH 7.2, non-sterile	Severn Biotech	20-7400-10
RPMI 1640	Gibco	21875-0
Stable Peroxidase Substrate Buffer 10x	Pierce	34062
Tissue culture 96 well flat bottom plates	Corning	BC015
Tween®20	Sigma	P1379-500mL

2.7.2 Antibodies

Table 2.10 Antibodies Used for ELISA

Specificity	Clone	Conjugate	Host species	Supplier	Code number
IgG Standard	--	--	Human	Sigma	I450
IgG	--	--	Goat	AbD Serotec	204001
IgG, Fcγ*	--	HRP	Goat	Jackson Immuno Research	109-036-098
IgG, heavy and light chains*	--	HRP	Goat	Jackson Immuno Research	109-036-088
IgG1	2C11	--	Mouse	AbD Serotec	5218-9850
IgG2	G18-21	--	Mouse	BD Pharmigen	555873
IgG3	HP6050	--	Mouse	AbD Serotec	MCA516G
IgG4	G17-4	--	Mouse	BD Pharmigen	555881
IgG Subclass Standard	--	--	Human (serum)	European Reference Materials	ERM-DA40k/IFCC

* $F(ab')_2$ fragment

2.7.3 Total IgG ELISA

IgG concentrations in B cell culture supernatants were determined by ELISA using the following method. First, the capture antibody, a goat anti-human IgG, was diluted in carbonate-bicarbonate buffer to a final concentration of 1 µg/mL. Next 100 µL of this coating solution was added to each well on a Maxisorp™ 96 well plate and incubated overnight at 4˚C. After overnight incubation, the coating solution was removed from the wells and 200 µL of blocking buffer, 2% Skim Milk/PBS + 0.5% Tween®20 (PBS-T), was added to each well. Plates were incubated for 2 hours and then wells were washed twice with 250 µL of PBS-T. Next, the IgG standard was diluted to 400 ng/mL in 50% RPMI 1640 media (same as cell culture media) and 50% PBS-T/1% Skim Milk (assay buffer) and serially

diluted 1:2 in the well plate down to 0.78 ng/mL in duplicate so that each well had a final volume of 50 µL. The remaining wells were given 25 µL of assay buffer and 25 µL of supernatants or diluted supernatants derived from B cell cultures. Standards and samples were incubated for 2 hours before wells were washed four times with 250 µL of PBS-T. Next the secondary antibody, goat anti-human IgG-HRP, was diluted 1:1000 in assay buffer and 50 µL of this solution was added to each well. After a two hour incubation period, wells were washed four times with 250 µL PBS-T. Next 50 µL of substrate, which was prepared by diluting 5 mg of OPD into 10 mL 1x Stable Peroxidase Substrate Buffer, was added to each well. The substrate was incubated for 15 minutes and the reaction was stopped by the addition of 50 µL of 1 M HCl to each well. The absorbance of each well was determined using the Flurostar Omega (BMG Labtech) Spectrophotometer using an absorbance of 492 nm and a reference wavelength subtraction of 650 nm. The standard curve fitting was performed using GraphPad Prism© software with a 4-parameter curve fit with no weighting using a minimum of 6 points on the standard curve (Findlay and Dillard 2007).

2.7.4 Subclass IgG ELISA

The composition of IgG subclasses (IgG1,2,3 and 4) in cell culture supernatants was determined by an ELISA which was developed for this purpose and modified from a method by Pinna et al. (Pinna, Corti et al. 2009). Wells on Maxisorp™ 96 well plates were coated with either mouse anti-human IgG1, IgG2, IgG3 or IgG4 antibodies in a carbonate-bi-carbonate buffer at a concentration of 1 µg/mL. After an overnight incubation at 4°C, coating solution was removed from the wells and

200 µL of 2% Skim Milk in PBS-T were added to each well. Plates were incubated for 2 hours and then wells were washed three times with 300 µL of PBS-T. Immunoglobulin values for each subclass were quantified using Certified Reference Material 470 (Schauer, Stemberg et al. 2003) as the assay standard. The IgG subclass standard was diluted to a concentration of 1280 ng/mL for each subclass in standard buffer: 50% RPMI media and 50% PBS-T/1% Skim Milk (assay buffer) and serially diluted 1:2 in standard buffer down to 5 ng/mL in duplicates, so that each well had a final volume of 50 µL. The remaining wells were given 25 µL of assay buffer and 25 µL of supernatants or diluted supernatants from B cell cultures. Standards and samples were incubated for 3 hours at 37˚C before wells were washed four times with 300 µL of PBS-T. Next, the secondary antibody goat anti-human IgG $F(ab')_2$ fragment (heavy and light chain specific), was diluted either 1:1000 (IgG1 and IgG2) or 1:5000 (IgG3 and IgG4) in assay buffer and 50 µL of this solution was added to each well. After 3 hour incubation at 37˚C, wells were washed four times with 300 µL of PBS-T. Next 50 µL of substrate, which was prepared by diluting 5 mg of OPD into 10 mL of 1x Stable Peroxidase Substrate Buffer, was added to each well. The substrate was incubated for 15 minutes before the reaction was stopped by the addition of 50 µL of 1 M HCl to each well. The absorbance of each well was determined using the Flurostar Omega (BMG Labtech) Spectrophotometer using an absorbance of 492 nm and a reference wavelength subtraction of 650 nm. The standard curve fitting was performed using GraphPad Prism© software with a 4-parameter curve fit using a minimum of 6 points on the standard curve.

2.7.5 Novel Cell-based ELISA

A cell-based ELISA was developed to detect the binding of antibodies to cell surface antigens (Figure 2.2). Adherent cells of interest were first plated onto flat bottom tissue culture 96 well plates at a density of 3 x10^5 cells in 200 µL of normal media and placed in a 37˚C incubator. Once cells were 80-100% confluent in all wells, the media were removed and wells were lightly fixed in 50 µL of 0.5% formaldehyde/HBSS, calcium and magnesium free, overnight or until the solution evaporated. Plates were then either wrapped in foil and placed in a freezer set to -80˚C or used immediately.

On the day of the assay, plates were thawed for 30 minutes if stored in the freezer and wells were washed 3 times with 250 µL of PBS and then blocked with 5% Skim Milk/PBS for 90 minutes. Wells were again washed 3 times with 250 µL of PBS. After washing, 50 µL of 1% Skim Milk/PBS were added to each well. Samples of interest were added to each well in 50 µL volumes and incubated for 90 minutes. For cell culture supernatant screening, positive and negative controls were added to each plate in triplicate along with samples. The negative control was a human IgG1 antibody and the positive control was Trastuzumab (Herceptin™), a human anti-Her2/neu antibody which has been shown to bind to melanoma cells (Stove, Stove et al. 2003) & (Figure 3.16). Both positive and negative control antibodies were tested at a concentration of 500 ng/mL. Plates were incubated for 90 minutes and wells were washed four times with 250 µL of PBS-T. The detection of IgG was performed with an anti-human IgG, F(ab')$_2$ antibody fragment, specific for Fcγ. This antibody fragment was diluted 1:500 in %1 Skim Milk/PBS-T buffer and 50 µL were added to each well. After 45 minute incubation, plates were washed four times in PBS-T. Next, 5 mg of OPD was diluted in 10 mL of 1X substrate buffer

and 50 µL volumes of this substrate solution were added to each well. After 15 minutes the enzymatic reaction was stopped by the addition of 50 µL of I M HCl to each well. Using the Flurostar Omega plate reader, the absorbance of each well was read at 492nm with reference wavelength subtraction of 650 nm. Corresponding B cell cultures with the highest absorbance relative to the positive and negative controls in this assay were selected for further expansion in *ex vivo* cultures (described further in Section 2.8.2).

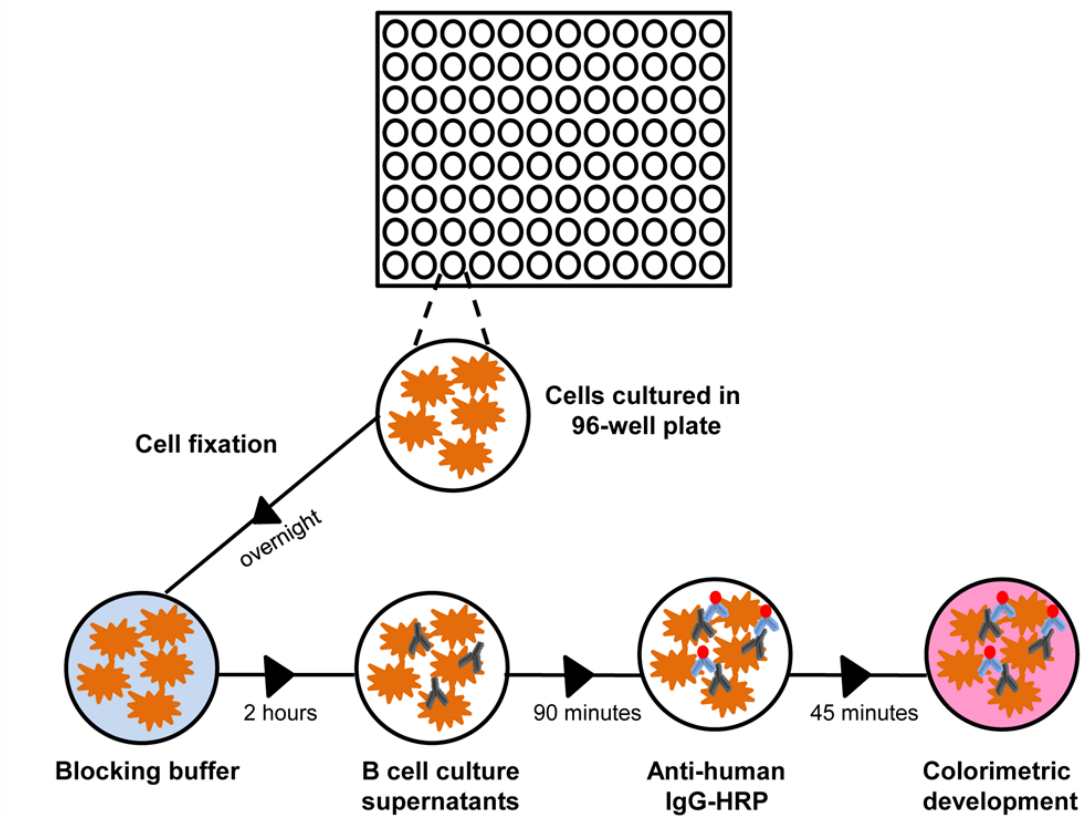


Figure 2.2 Schematic of cell-based ELISA used to detect antibodies against cell antigens. Depiction of the steps used to detect the binding of antibodies to cell surface antigens by ELISA.

2.8 Assessment of Antibody Reactivity to Tumor Cells

2.8.1 Criteria for Evaluating Antibody Responses to Melanoma

IgG antibodies secreted in human *ex vivo* B cell cultures derived from both patients and health volunteers were assessed for reactivity against cells of interest using the cell-based ELISA described previously (see Section 2.7.5). The reactivity of the supernatant from each B cell culture to tumor cells or melanocytes relative to a negative control antibody, non-specific human IgG, was evaluated. In order to compare anti-tumor antibody responses to metastatic and primary melanoma cells between patients and healthy volunteers and among patient groups, absorbance values were normalized against a negative control using the following formula:

$$Fold\ change = \frac{Absorbance\ of\ B\ cell\ culture\ supernant}{Mean\ absorbance\ of\ non\text{-}specific\ IgG1}$$

This calculation was also used to normalize ELISA results across multiple melanoma cell lines and primary melanocytes in order to evaluate the tumor specificity of antibodies.

2.8.2 Selection of Clones

To compare the percentage of positive melanoma-reactive cultures across patients, absorbance values were also normalized against the positive control. Others have considered tumor-reactive antibody cultures with absorbance values above negative controls + 3 standard deviations as positive cultures (Punt, Barbuto et al. 1994; Zhang, Lake et al. 1995; Zhang, Casiano et al. 2003). However, this approach

used more stringent criteria through the use of Trastuzumab as a positive control antibody as a means to identify cultures reactive to melanoma cells (refer to the Appendix for a comparison of these two approaches). For these evaluations, the mean positive antibody control absorbance was assigned a relative absorbance of 1 for each plate and B cell cultures were converted from absorbance units to relative absorbance units. Next, culture wells with relative absorbance values greater than 0.75 to melanoma cells but not to melanocytes were then labeled positive cultures.

Following the selection of promising clones from positive cultures, i.e. wells with high reactivity to melanoma cells and not melanocytes, monoclonal cultures were derived following the cloning of cells by serial dilution of B cells in 96 well plates (Figure 2.3). This conservative approach was chosen over other single cell cloning approaches such as single cell sorting by FACS and limiting dilution because of the maintenance of antibody clones at higher densities should the cell plating efficiency be low.

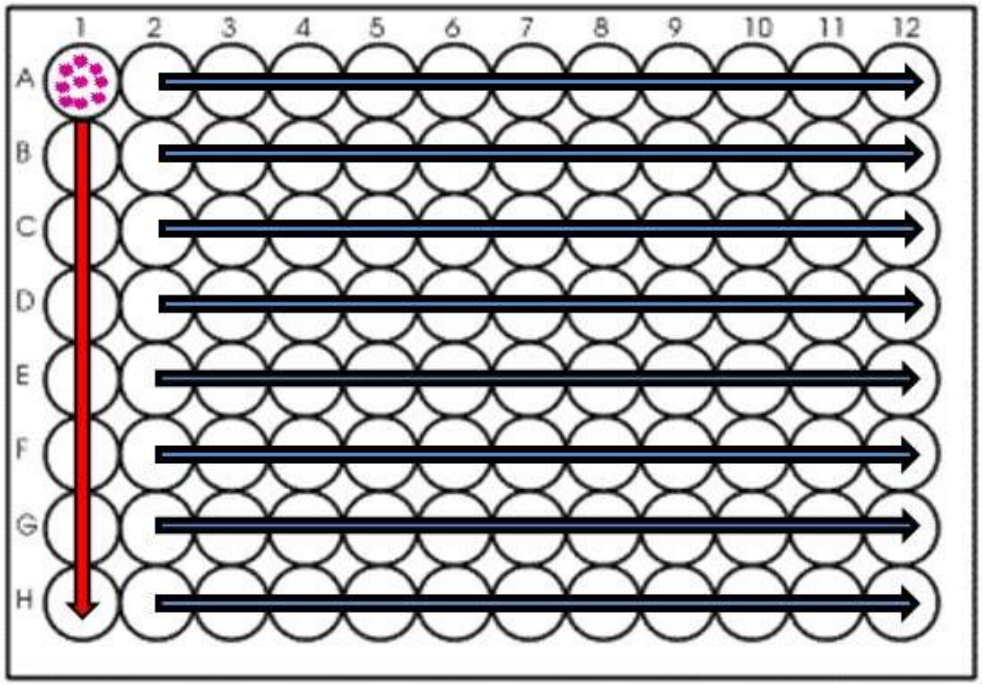


Figure 2.3 Derivation of monoclonal cultures by serial dilution. Following the selection of melanoma-reactive antibody cultures, B cell clones from a chosen culture were plated at a density of 1 x 10^3 cells in well A1 on 96 well round bottom plates. The rest of the plate wells were filled with 200 µL of RMPI warmed to 37˚C. First, well A1 was thoroughly mixed and 100 µL were removed from this well and serially diluted (1:2) from column A from row 1 down to row 8. Next using a multichannel pipette, all of the wells in column A were thoroughly mixed and then 100 µL were serially diluted (1:2) across the plate from column A to column H. After waiting 2 to 3 hours for cells to settle to the plate bottoms, wells were then checked by light microscopy to identify cultures with single cells. All wells then received previously frozen irradiated autologous PBMCs at plated at density of 1 x 10^5 cells in 100 µL of RMPI at 37˚C. All culture wells were maintained and identified single cell cultures were monitored for clonal expansion by light microscopy and re-tested for reactivity to melanoma cells by flow cytometric methods or by the cell-based ELISA.

2.8.3 Limiting Dilution Assay (LDA)

The frequency of B cells producing melanoma-reactive IgG antibodies was estimated using LDAs. B cells were isolated from patients and plated at multiple densities (125, 500, 1,000, 1,500, 2,500, and 10,000 B cells/well) and cultured as described in Section 2.4.2. After 18 days in culture, the reactivity of B cell culture supernatants to melanoma cell lines and to normal melanocytes was evaluated using the cell-based ELISA. The percentage of B cell cultures that were non-reactive or negative for reactivity (with absorbance values <75% of the positive control) against the chosen cell line was then calculated. The frequency of melanoma-reactive IgG B cells was next estimated according to Poisson distribution when 37% of the wells were negative for reactivity to melanoma cells. Linear regression analysis was performed to extrapolate the point at which the regression line crossed the Y axis=37%. These calculations were done in line with the work of Pinna et al. who estimated the frequency of influenza-specific antibodies from *ex vivo* cell cultures (Pinna, Corti et al. 2009). It is important to note that LDAs performed herein are not against one specific antigen but rather are against cells. This means that the LDAs estimate reactivity of antibodies in cultures against various antigens on cells such as melanocytes, primary melanoma and metastatic melanoma. Due to nature of the methodology, it is possible that reactive/positive cultures may have arisen from more than one B cell clone. Thus the frequency of reactive antibodies to multiple antigens on various human cell types rather than the frequency of antibodies specific to a single antigen was estimated in these assays.

2.9 Cell Adhesion and Migration Assays

The ability of antibodies targeting HMW-MAA to restrict tumor cell adhesion and migration was evaluated using the general reagents (Table 2.11) and antibodies (Table 2.12) listed below.

2.9.1 General Reagents

Table 2.11 Reagents Used in Cell Adhesion and Migration Assays

Name	Supplier	Code number
96 Well flat bottom plate	Corning	BC015
Bovine serum albumin (BSA), tissue culture grade	Sigma	A9418
Cell stain	CHEMICON	90144
Cotton swabs	CHEMICON	10202
Crystal violet	Sigma	HT90132
DMEM	Gibco	41966-029
Ethanol	BDH Prolabo	20821.330
Extraction buffer	CHEMICON	90145
Fibronectin	Sigma	F2006
PBS, sterile	Gibco	20012
Sterile 24 well plates with collagen-coated Insert, 8μM pore size	CHEMICON	90248

2.9.2 Antibodies

Table 2.12 Antibodies Used for Cell Adhesion and Migration Assays

Specificity	Clone	Species specificity	Host species	Source
HMW-MAA	225.28S IgG and IgE classes	Human	Chimeric (mouse/human)	Antibodies were engineered by Tihomir Dodev, KCL from a published sequence [a]
FRα	MOv18 IgG and IgE classes	Human	Chimeric (mouse/human)	Antibodies were engineered by Tihomir Dodev, KCL from a published sequence [b]

[a] Neri, Natali et al. 1996; [b] Gould, Mackay et al. 1996

2.9.3 Cell Adhesion Assay

Inhibition of melanoma cell adhesion by monoclonal antibodies was measured *in vitro* using a cell adhesion assay (Wang, Osada et al. 2010). Prior to starting the assay, A-375 melanoma cells were allowed to reach 80% confluence in culture and then grown in serum-free DMEM media for 24 hours. On the day of the assay, a 96 well plate was coated with fibronectin at a concentration of 6 µg/mL, with the exception of one row of wells which was left uncoated. These uncoated wells were given 100 µL of 1% BSA to serve as a negative control of adhesion for the assay. At the same time, serum starved A-375 cells at a concentration of 2 $x10^6$ cells/mL were incubated with PBS alone or with a buffer containing the following antibodies: IgG or IgE class antibodies against HMW-MAA and MOv18 IgG or IgE antibodies recognizing the tumor associated antigen FRα (Gould, Mackay et al. 1999); these MOv18 antibodies which do not react with A-375 melanoma cells and therefore served as negative isotype control antibodies. Antibodies were used at a concentration of 0.25 mg/mL in PBS and allowed to interact with A-375 melanoma cells for 20 minutes at room temperature prior to the addition on the fibronectin or BSA coated wells.

After one hour incubation at 37˚C with fibronectin or 1% BSA, the 96 well coated plate was washed with room temperature PBS. Washing was performed by gently adding 200 µL of PBS to the side of the well using a multichannel pipette. Immediately following washing, 2 x 10^5 cells incubated with appropriate antibodies as described above were seeded into the fibronectin or BSA coated 96 plate wells and plates were placed in an incubator set at 37˚C, 5% CO_2 for 30 minutes. After 30 minutes, wells were washed with 200 µL of PBS five times to remove any non-adherent cells. Wells were then given 50 µL of 70% ethanol to fix

any adherent cells. Next, ethanol was removed from wells and cells were stained with 50 µL 0.1% crystal violet diluted in water for 30 minutes. Wells were then washed twice in tap water and resuspended in 50 µL of PBS and the absorbance of each well at 540 nm was read using the Flurostar Omega plate reader. Results were expressed as percent inhibition of adhesion relative to the PBS-treated control.

2.9.4 Collagen-based Cell Migration Assay

The ability of anti-HMW-MAA IgG and IgE antibodies to inhibit the migration of A-375 melanoma cells across a collagen layer *in vitro* was evaluated using the QCM™ 24-Well Collagen-Based Cell Invasion Assay (Chemicon, Catalog number ECM 551). This collagen layer was comprised of approximately 85% type I collagen and 15% type III collagen. A-375 cells were grown until 80% confluent and then placed in serum free DMEM medium for 24 hours prior to the assay. On the first day of the assay, reagents were first brought to room temperature and media warmed to 37˚C. Next, collagen coated invasion chambers (inserts) were rehydrated with 300 µL of pre-warmed serum-free media for 15 to 30 minutes. After rehydration, media was carefully removed from each insert. A-375 cells at a concentration of 1 x 10^6 cell/mL were incubated at room temperature with antibodies at 0.25 mg/mL or PBS for 20 minutes and then 250 µL of this prepared suspension was added to the appropriate inserts. Next, 500 µL of media with 10% FBS per well were added to the lower chambers on the 24-well plate, except for negative control wells; each of the latter wells received 500 µL of media without FBS. Plates were covered and incubated at 37˚C, 5% CO_2 for 24 hours.

On the second day of the assay, cells/media were carefully removed from the top side of the insert by pipetting out the remaining cell solution. The invasion chamber was then inserted into a clean well on a 24 well plate containing 400 µL of cell stain solution and incubated for 20 minutes so that migrated cells could be identified. The invasion chamber was next dipped into a beaker of tap water and rinsed several times. While the chamber was still moist, a cotton swab was used to gently remove the non-invading cells from the interior of the chamber with careful attention made not to puncture the membrane and to remove cells from the inside perimeter. This step was then repeated with another clean cotton swab. The stained chamber was then transferred to a clean well containing 200 µL of extraction buffer for 15 minutes, and the stained cells were extracted from the underside of the chamber by gently tilting the chamber back and forth several times throughout the incubation. Chambers without cells were also processed as described above and used as blanks to calculate assay background. The chambers were removed from the plates and 100 µL of the dye mixture was transferred to a 96 well plate. Absorbance was next read at 560 nm using the Flurostar Omega plate reader. Results were expressed as percent inhibition of migration relative to the PBS control.

2.10 Cytotoxicity Assays

Cytotoxicity assays were employed to measure the ability of patient-derived and engineered monoclonal antibodies to mediate cellular cytotoxicity against tumor cells. The general reagents (Table 2.13) and antibodies (Table 2.14) used in these assays are listed in the following tables.

2.10.1 General Reagents

Table 2.13 Reagents Used in Cytotoxicity Assays

Name	Supplier	Code number
Carboxyfluorescein diacetate,succinimidyl ester (CFSE)	Sigma	21888
CellTracker™ blue dye (7-Amino-4-Chloromethylcoumarin)	Molecular Probes	C2110
4',6-diamidino-2-phenylindole (DAPI)	Invitrogen	D1306
DMEM	Gibco	21063029
LIVE/DEAD Viability/Cytotoxicity Kit for Mammalian Cells containing Calcein AM and Ethidium homodimer-1	Molecular Probes	L3224
Neuraminidase, Type X	Sigma	N-2133
6 well tissue culture plates	Corning	3516

2.10.2 Antibodies

Table 2.14 Antibodies Used in Cytotoxicity Assays

Specificity	Clone	Conjugate	Host species	Supplier	Code number
CD89	A59	PE	Mouse	BD Biosciences	555686
HMW-MAA	225.28S IgG and IgE classes	--	Chimeric	KCL	Antibodies were engineered by Tihomir Dodev from a published sequence [a]
FRα	MOv18 IgG and IgE classes	--	Chimeric	KCL	Antibodies were engineered by Tihomir Dodev from a published sequence [b]

[a] Neri, Natali et al. 1996; [b] Gould, Mackay et al. 1996

2.10.3 Live/Dead Cell Imaging Assays

The tumor-killing potential of patient derived monoclonal antibodies was assessed using a three-color fluorescent live cell imaging cytotoxicity assay. A-375 melanoma cells were plated overnight at a density of $2x10^5$ cells per well on 6 well culture plates. Using the components of a LIVE/DEAD® Viability/Cytotoxicity kit, immediately prior to cytotoxicity assays tumor cells were labeled with 2μM of Calcein AM, a green fluorescent dye which labels viable cells but is quenched upon loss of cell viability (Lichtenfels, Biddison et al. 1994). A-375 cells were incubated with Calcein AM for 30 minutes in RPMI 1640 media free of phenol red and FCS. Cells were then washed in RPMI 1640 supplemented with 10% FCS and 1% PenStrep, and resuspended in media containing 4 μM Ethidium homodimer-1. Ethidium homodimer-1 incorporates into the DNA of dead cells which have lost

their membrane integrity and served as a label for cell death in this assay (King 2000). U-937 monocytic cells expressing Fcγ receptors were used as immune effector cells at a ratio of 3:1 (effectors: tumor cells) (Bracher, Gould et al. 2007). Immune effector cells were incubated with antibodies for 30 minutes, stained with the CellTracker™ blue dye, washed and added to the Calcein AM-labeled tumor cells in culture wells containing Ethidium homodimer-1. Samples were incubated and images were captured every 5 minutes for a period of two hours in a humidified temperature-controlled chamber using a Zeiss Axiovert (20x objective) microscope and AxioVision software system (Carl Zeiss, Jena, Germany). Following incubation, fluorescent intensities of Calcein AM-positive live tumor cells, as well as incorporation of Ethidium Homodimer-1 into cells were measured (Table 2.15) and cell death was assessed with NIS-Elements BR 3 software (Nikon). The movement of effector cells in the cultures was tracked and analyzed using Imaris software (Bitplane, Zurich, Switzerland).

Table 2.15 Spectral Properties of Dyes Used in Cytotoxicity Assays

Dye	Color	Excitation maximum (nm)	Emission maximum (nm)
Calcein AM	Green	495	515
CellTracker™ blue dye	Blue	353	466
CFSE	Green	494	521
DAPI	Blue	358	461
Ethidium homodimer-1	Red	495	635

2.10.4 ADCC and ADCP Assays

The ability of antibodies to induce tumor cell death by cell-mediated cytotoxicity (ADCC) and/or phagocytosis (ADCP) was evaluated using a three-color flow cytometric method (Bracher, Gould et al. 2007). Assays were performed as described by Bracher et al. with the exception of the labels used for tumor and dead cells along with the use of freshly isolated human monocytes from patients and healthy volunteers rather than cell lines. The day before the assay A-375 cells were incubated with 5.0 μM CFSE in PBS for 10 minutes at 37˚C. Cells were then washed in DMEM containing 10% FCS and returned to normal cell culture conditions for this cell line overnight.

On the day of the assay, primary monocytes were washed in PBS/2% FCS, 1 mM EDTA (monocyte isolation buffer) and incubated with neuraminidase at concentration of 0.3 units per 2×10^7 cells in 1 ml PBS plus 2% human AB serum/DMEM which ensured retained expression and availability of FcεRI receptors on the surface of human monocytes as previously reported (Reischl, Corvaia et al. 1996; Karagiannis, Wang et al. 2003). After 30 minute incubation, monocytes were washed once with monocyte isolation buffer. Next, CFSE-labeled tumor cells were washed in FACS buffer and mixed with freshly isolated (within one hour of cell isolation) human monocytes (see Section 2.3.4) at an effector to target ratio (E:T) of 3:1. Antibodies were diluted to 10 μg/mL in PBS and this or PBS alone was added to tumor and effector cell mixtures and incubated for 3 hours at 37˚C and 5% CO_2. Cells were than washed in sterile FACS buffer and human monocytes were next incubated for 30 minutes at 4˚C with an anti-CD89-PE antibody, which targets the IgA Fc receptor (FcαRI) expressed on monocytes.

Following monocyte labeling and the washing of cells once in FACS buffer, dead cells were then stained with 5 μg/mL of DAPI (emission max=452 range=410-600 nm) replacing the use of propidium iodide (emission max=650; range 600-750nm)(Bracher, Gould et al. 2007). This modification allowed reduced spectral overlap between DAPI (emission max=452 range=410-600 nm) and CSFE labeled cells (emission max=520nm; range 480-650) in the flow cytometer and represents an improvement from a previously published method. Cells were then washed and mixed thoroughly to interrupt cell-cell contact, and samples were resuspended in 300 μL of FACS buffer and analyzed on a FACS Canto (BD Biosciences) flow cytometer using FACSDIVA™ software (BD Biosciences) for data acquisition and analysis.

ADCP was measured by calculating the number of cells positive for both tumor cells (CFSE, read in FITC channel) and effector cells (PE) (see Figure 2.4 left, quadrant 2 [Q2]) relative to controls not treated with antibodies. ADCC was calculated from the number of dead tumor cells (see Figure 2.4 right, P1 gate) stained with DAPI (Pacific Blue) along with the total loss of tumor cells in the assay (see Figure 2.4, quadrants 2 and 3) to account for lysed cells; both numbers were calculated relative to tumor and effector cells not treated with antibodies.

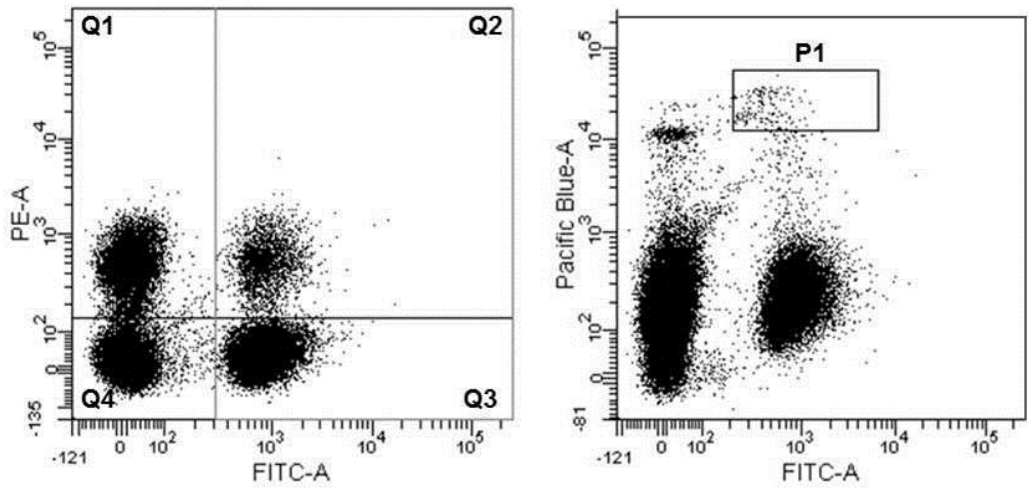


Figure 2.4 Quantification of ADCP and/or ADCC by flow cytometry. Antibody mediated phagocytosis was calculated from dot plots (left) with melanoma cells stained in green (FITC, x-axis) and monocytes in red (PE, y-axis). Phagocytosis was estimated from cells that were double positive for FITC and PE dyes (Q2). Cytotoxicity was calculated from dot plots (right) where the number of dead melanoma cells was estimated by FITC and Pacific Blue double positive cells (P1) along with loss of total melanoma cells (right) from Q2 and Q3 compared to cells treated with no antibody. See below for exact calculations.

The following formulas were used to calculate antibody mediated tumor cell death:

$$\%\ ADCC\ = \frac{X + P1}{Control\ Q2 + Q3} \times 100$$

(Q2 + Q3)= Number of FITC stained tumor cells in assay
(Control Q2 +Q3)= Mean number of FITC stained tumor cells from tubes (n=3) given no antibody
X= (control Q2 + Q3)-(Q2+Q3)
P1= Number of cells stained with DAPI

$$\%ADCP = \frac{Q2}{Control\ Q2 + Q3} \times 100$$

2.11 Statistical Methods

All statistical analyses were performed using GraphPad Prism software (version 5.03, GraphPad, San Diego, CA).

2.11.1 Assessments of Anti-tumor Antibodies Produced from Peripheral Blood B cells

Descriptive statistics were generated to examine the distribution of melanoma-reactive B cell cultures from each patient including the mean, 95% CI and maximum reactivity to melanoma cells. A two-sided Student's *t* test was used to compare the mean reactivity of antibody cultures derived from melanoma patients, with those values derived from healthy volunteers against primary or metastatic melanoma cell lines. A two-sided Student's *t* test was also used to compare antibody responses between patients with non-metastatic and metastatic disease. A one-way analysis of variance (ANOVA) was used to compare antibody reactivity to a metastatic melanoma cell line among B cell cultures derived from patients with Stage II, III and IV disease using a Tukey's post hoc comparison test.

2.11.2 Analysis of Peripheral Blood Memory B cell Compartment

The Mann-Whitney U test was used to compare the peripheral blood memory B cell compartment between healthy volunteers and metastatic melanoma patients or between non-metastatic melanoma patients and healthy volunteers.

2.11.3 Analysis of the Proportional Production of IgG Subclasses from Peripheral Blood and Tumor-resident B cells

The proportion of IgG1-4 subclasses secreted in *ex vivo* B cell cultures was compared between healthy volunteers and patients with metastatic melanoma using a two-sided Student's *t* test.

2.11.4 Cell Adhesion and Migration Assays

A two-sided Student's *t* test was used to compare the inhibition of tumor cell migration and adhesion with the addition of tumor-specific monoclonal antibodies compared to non-specific antibody isotype controls.

2.11.5 Cell-mediated Cytotoxicity Assays

A two-sided Student's *t* test was used to compare antibody-mediated tumor cell killing between tumor-specific and non-specific monoclonal antibodies derived from the same patient. A two-sided Student's *t* test was also employed to compare the movement of immune monocytic effector cells, pre-incubated with patient-derived antibodies, in contact with tumor cells with the movement of immune cells not in contact with tumor cells. For ADCC/ADCP assays, a two-sided Student's *t* test was also employed to compare anti-HMW-MAA IgG and IgE mediated phagocytosis and cytotoxicity to either isotype control antibodies or no antibody controls.

Chapter 3: A Novel Approach for Identifying Anti-melanoma Antibodies from Clinical Specimens[3]

[3] Sections 3.2 & 3.3 and Figures 3.1-3.3, 3.6-3.8, 3.13-3.16, and 3.21 are produced in part or full from Gilbert, Karagiannis et al. 2011.

3.1 Introduction and Aims

Monoclonal antibodies have emerged as a key therapeutic modality in the treatment of many cancers (Pillay, Gan et al. 2011). The recent approval of the first monoclonal antibody for melanoma, Ipilimumab, has shown promising clinical results including it being the first melanoma drug to significantly prolong survival (Hodi, O'Day et al. 2010; Prieto, Yang et al. 2012). The achievement of such profound clinical success of Ipilimumab is in part due to the extensive understanding of the nature of T cell responses in melanoma, which highlights the merits of understanding and potentially harnessing the host immune responses to cancer in order to develop efficacious therapeutics, including monoclonal antibodies that can activate the immune system. While there has been much focus on T cell-mediated immune responses in melanoma, limited knowledge exists on the role B cells may play in melanoma and the nature and potency of the antibodies they produce.

There has been some focus on the identification of novel antibodies against TAAs from the *in vivo* study of humoral immune responses to cancer cells in both mice and individuals with cancer. Melanoma and some other cancers are thought to be immunogenic, with demonstrated serological antibody responses to TAAs (Sahin, Türeci et al. 1995; Old and Chen 1998; Stockert, Jäger et al. 1998). Approaches to discovering such novel antibodies have included the generation of antibodies by the immunization of mice with human tumor cells/cell lysates/antigens to generate hybridoma cell lines or in some cases phage libraries; the immunization of patients with genetically modified autologous tumor cells with enhanced immunogenicity; and the isolation of antibodies from B cells located in the peripheral blood or tumors from individuals with cancer (Kirkwood and Robinson

1990; Chester, Begent et al. 1994; Cai and Garen 1995). These approaches can all lead to the identification of novel antibodies against known or unknown antigens; however, some approaches require rather time-consuming processes, such as the immunization of animals and generation of hybridoma cells lines, the creation of phage display libraries and the selection of clones, or *in vitro* affinity maturation, and these processes are often followed by the engineering of scFv into full-length humanized or human antibodies. Studying the humoral immune response of individuals with cancer harbors the potential to rapidly identify novel human affinity-matured antibodies, with potentially high specificity to TAAs. Potential benefits of this approach to discovering antibodies from patients include the identification of antibodies against clinically relevant tumor antigens with naturally matched heavy and light chains and the elimination of the requirement for *in vitro* affinity maturation to antigen. Additionally, the fully human sequences of patient antibodies are also expected to be associated with reduced concerns of immunogenicity when administered in humans, compared to engineered antibodies which often contain murine variable regions.

While identifying tumor-specific antibodies from patients themselves may allow for the selection of relevant antibodies generated by the host in response to malignancy, in reality the approach poses several technical challenges. Primarily, a major concern lies with the production of sufficient amounts of antibodies from B cell cultures derived from patients. To create long term primary cell cultures, B cells can be 'immortalized' with EBV, a lymphotrophic herpes virus, which has historically been used to establish antibody producing B cell lines from melanoma patients (Watson, Burns et al. 1983; Kirkwood and Robinson 1990). However, B cell transformation with EBV alone has been limited by low cloning efficiency,

reported to be as low as 3% (Sugden and Mark 1977). Past efforts to produce antibody-secreting B cell cultures from patients with melanoma using EBV transformation cite the loss of antibody production from these EBV transformed B cells as a major technical obstacle in the identification of antibodies against melanoma-specific antigens from patient B cells (Kirkwood and Robinson 1990; Yeilding, Gerstner et al. 1992).

The creation of antibody-secreting B cell lines from melanoma patients pre-date more recent improvements to activate B cells in culture. One such major improvement to the culturing of patient B cells has been reported by Traggiai et al., who demonstrated that the addition of CpG 2006, a TLR9 agonist and polyclonal activator of memory B cells, to EBV transformed B cells increased the cloning efficiency rate of memory B cells to 30-100% (Traggiai, Becker et al. 2004). The addition of CpG 2006 resulted in a major improvement to the historically low cloning efficiency rates of human B cells. The technique presented by Traggiai and colleagues has permitted novel insights into humoral memory responses to infectious disease by examining the antibody repertoire of an individual following infection with SARS coronavirus. This advancement in *ex vivo* culturing and activation of B cells has yet to be applied to the field of cancer and if it is possible to achieve this, a greater understanding of humoral immune responses to cancer can be gained. Thus, applying this technique to individuals with melanoma could allow for closer examination of host B cell responses to melanoma as well as for the identification of novel melanoma-specific antibodies. The aim of the work described within this chapter is to apply the methodology set forth by Traggiai and colleagues to individuals with melanoma as an approach to discover human melanoma-specific antibodies.

Crucial to performing a comprehensive screening of the specificity of antibodies produced by individuals' B cells is the construction of an inexpensive medium-throughput assay as a means to identify melanoma-specific antibodies from B cell culture supernatants. Screening antibodies against whole cells, rather than recombinant antigens or antigenic peptides, provides a number of advantages. Firstly, antibody reactivity can be assessed against the multitude of antigens expressed on tumor cells, thus permitting an assessment of the broad anti-tumor mature B cell responses to melanoma. Secondly, using whole cells rather than a few pre-defined recombinant antigens may allow for the identification of antibodies against novel antigens. Thirdly, while melanoma cells are readily available, very few cell surface antigens in melanoma have been identified and are available commercially in recombinant forms at the present time. Finally, antigens on the surface of melanoma cells are expected to be expressed in their native and active conformation and this may not always be the case for antigens expressed in recombinant forms. For these reasons, a cell-based screening methodology was devised and verified to serve as a crucial tool in the identification of novel melanoma-reactive antibodies.

Applying new methods to activate B cells *ex vivo* will allow for evaluation of the humoral memory responses to melanoma to complement serological studies (Chapter 4), and such evaluations may reveal novel antibodies against tumor associated antigens (Chapter 5). Instrumental to these evaluations is the development of a screening methodology to identify melanoma-reactive antibodies; this, along with the evaluation of the feasibility of identifying monoclonal antibodies from melanoma patient *ex vivo* B cell cultures, remains a primary focus of this chapter.

The objectives of this chapter are threefold:

1. To establish *ex vivo* human B cell cultures from healthy individuals and from patients with cancer capable of secreting large amounts of IgG antibodies for further evaluations.
2. To develop a robust and medium-throughput methodology to detect antibodies from human B cell culture supernatants that react to tumor cells.
3. To apply the above newly developed screening methodology to perform initial feasibility assessments of the reactivity of human-derived antibodies to tumor cells.

3.2 Establishment of Antibody-secreting Cultures from Human B cells

As a first step in the evaluation of antibodies from clinical specimens and their reactivity to melanoma cells, the isolation and expansion of B cells in culture was assessed in order to demonstrate that B cell cultures could be established from healthy individuals and from patients with cancer. Additionally, the quantities of IgG antibodies secreted by these B cells were measured to ascertain that these B cells were capable of producing sufficient quantities of IgG to further study the reactivity of these antibodies to cell surface antigens.

3.2.1 Expansion of Human B cells *ex vivo* from Peripheral Blood

With the aim of establishing antibody-secreting human *ex vivo* cell cultures, B cells were isolated from the peripheral blood unfractionated PBMCs of human volunteers using negative selection. Following negative selection (detailed previously in Chapter 2, Section 2.3.3), mature B cells (CD22+ cells) were isolated in high purity (>90%). Low populations of both CD3+ T cells (Figure 3.1, left) and CD14+ monocytic cells (Figure 3.1, right) were measured to be present following B cell isolation, with a representative example shown to be comprised of 0.3% CD3+ T cells and 2.1% CD14+monocytic cells. The measurement of low levels (<5%) of the two most abundant immune cell populations in PBMCs (i.e. T cells and monocytic cells), demonstrate that negative selection was an adequate method to isolate relatively pure and large populations of mature B cells from volunteer peripheral blood and would provide sufficient amounts of B cells from humans for the purposes of this work .

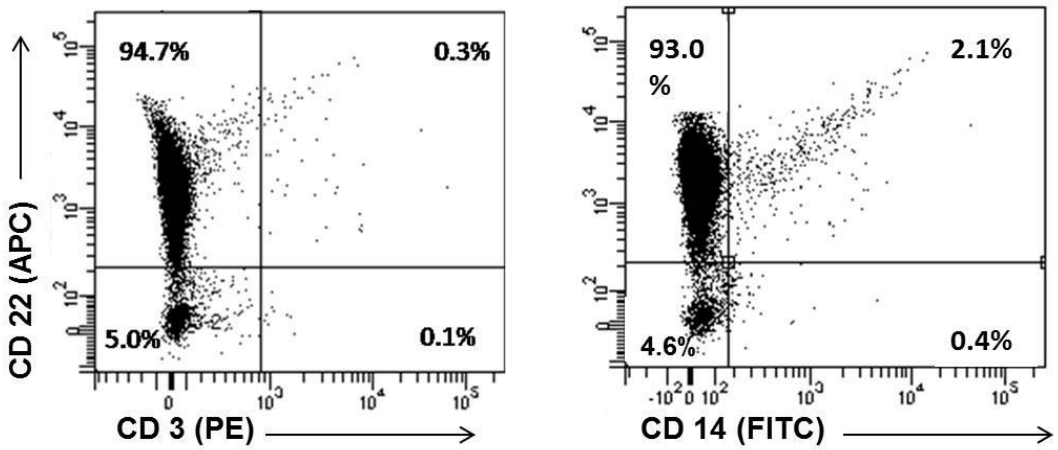


Figure 3.1 Purity of human B cells for primary cell culture. Human B cells were isolated from blood by negative selection using RosetteSep® and a Ficoll density gradient. Purity of B cell isolation was measured by comparing the amount of mature B cells (CD22+ cells) to other leukocytes such as T cells (CD3+, left) and monocytes (CD14+, right) using flow cytometry. Representative dot plots are shown.

While other methods of primary cell isolation, such as immunomagnetic or FACS cell sorting, may yield higher purities of B cells, negative selection using the Rosette Sep® was chosen due to efficiency in speed, minimal requirement for equipment, and minimal manipulation of cells. An important criterion in choosing this isolation methodology was the selection of a technique with least amount of handling of primary cells in order to obtain good cell viability in *ex vivo* cultures. By employing negative cell selection using Rosette Sep®, cells can remain within the sterility of a cell culture hood in a 50 mL conical tube, whereas other techniques require the transfer of cells to different instruments where cells undergo further manipulation such as binding to magnetic beads or flowing through a cytometric sorter. The introduction of extra steps or use of instrumentation not kept in a sterile environment during the isolation of primary cells can pose a challenge in the recovery and culture of primary cells due to the

introduction of possible contaminants which can reduce the viability of primary cell cultures for longer periods of maintenance (>1 month). In summary, the chosen approach to isolate human B cells was selected since it resulted in highly pure populations of mature B cells with minimal cell manipulation for establishing longer-term *ex vivo* B cell cultures.

Next, the expansion of *ex vivo* peripheral blood B cells in culture was monitored. B cell colonies were observed to form in the first 10 days and were viable for more than 8 weeks in culture (Figure 3.2). While B cells were initially cultured with feeder cells, EBV, and a TLR9 agonist, B cell colonies were observed to expand following subcloning without the addition of feeder cells, additional nutrients, or growth factors beyond those contained in the normal media. This antigen-independent sustained growth may be attributed to the differentiation of B cells into plasma blast and plasma cells driven by the addition of a TLR9 agonist which selectively results in the expansion of memory B cells (Bernasconi, Traggiai et al. 2002). The driving of B cells toward plasma blast and plasma cell phenotypes resulting in the secretion of high levels of antibodies could also impact cell proliferation and the long term division of these cells and their culture maintenance over longer periods of time. The expansion of B cells in *ex vivo* cultures was observed to be maintained over 8 weeks, which, for the purposes of this work, was sufficient time to allow for the identification of melanoma-reactive antibodies from cancer patients.

Day 1 **Day 10** **Week 8**

Figure 3.2 Expansion of human B cells in culture seen by light microscopy. Transformed and activated B cells were plated with autologous PBMCs after isolation (left), clonal formation was observed after 10 days (middle) and cells proliferated for 8 weeks or longer in culture (right) (40x magnification).

Cultured B cells were next evaluated to confirm the *ex vivo* expansion of switched memory B cells in culture. It is thought that TLR9 agonists preferentially expand memory B cells in culture because the upregulation of TLR9 receptors requires the triggering of B cell receptors and such receptors are present on memory B cells and not on naïve B cells (Bernasconi, Onai et al. 2003). In order to verify that the expanded B cells were memory B cells, expression of class switched antibodies such as those of the IgG class, indicative of the presence of memory B cells, was examined in patient cultures. B cells from 3 different melanoma patients comprised of 180 different B cell cultures (n=60 cultures per patient) were studied for intracellular and extracellular IgG expression after 18 days in culture by flow cytometry (method detailed in Chapter 2, Sections 2.6.3 and 2.6.4). Approximately 80% of the cultured B cells studied from these patients were found to be positive for IgG antibodies by intracellular and extracellular flow cytometric analysis (Figure 3.3, left). Additionally, to assess the presence of B cells producing other antibody classes, the reactivity of a $F(ab')_2$ (specific for heavy and light chains on

IgG, IgA, and IgM) was tested using B cells from the same patients. Approximately 88% of the cells were measured to be positive for these three antibody classes (Figure 3.3, right). One could estimate that 8% of the *ex vivo* cultured B cells expressed IgM and/or IgA antibodies, a small proportion of expanded B cells. In summary, IgG-positive B cells were found to expand in high percentages in *ex vivo* patient B cell cultures.

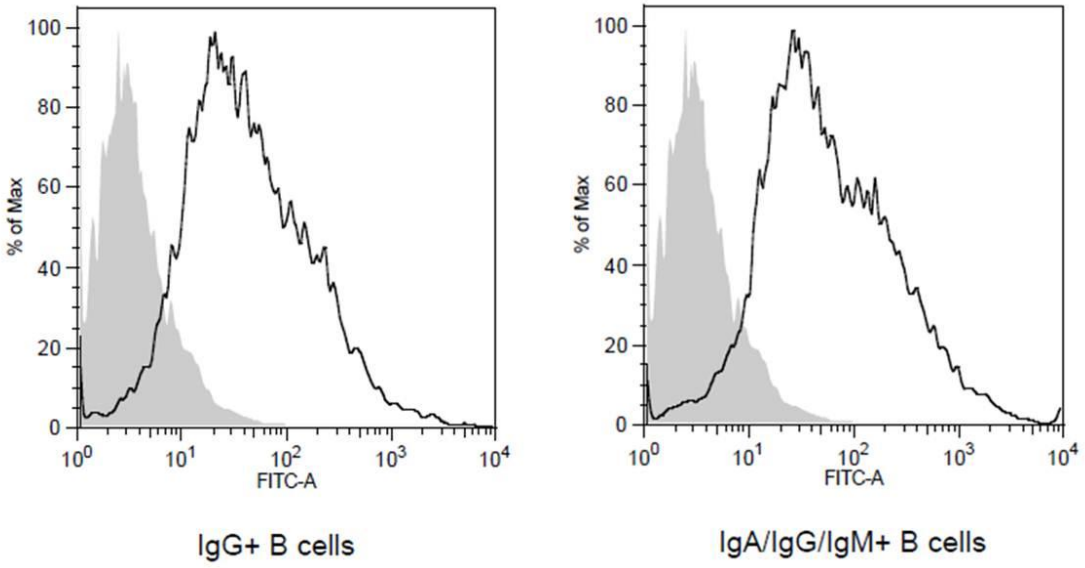


Figure 3.3 B cell expression of IgG in melanoma patient B cell cultures. Proportion of IgG positive (left, 80%) and IgA/IgG/IgM positive (right, 88%) B cells from the pooled B cell cultures (n=180) derived from melanoma patient peripheral blood (n=3). B cells were cultured for 18 days and IgG positive cells were identified (left) by intra and extracellular flow cytometric staining with a goat anti-human FITC-conjugated antibody $F(ab')_2$ fragment (solid black line) relative to a goat IgG-FITC $F(ab')_2$ fragment which served as isotype control (shaded grey). IgA/IgG/IgM positive B cells were also identified (right) using a FITC labeled $F(ab')_2$ fragment (solid black line) that reacts with IgA, IgG and IgM positive cells were also calculated relative to a goat IgG- FITC $F(ab')_2$ fragment isotype control (shaded grey).

The production of IgG from memory B cells is desirable over the IgM immunoglobulin classes since antibodies from these cells have been affinity matured and are expected to yield antibodies with high specificity to their antigen, thereby possessing greater potential clinical utility. Additionally, IgG is the dominant class of antibody used therapeutically for cancer due to its well described favorable pharmacokinetics, immunomodulatory properties, antigen specificity, and low incidence of off target toxicities (Weiner, Surana et al. 2010). The high expression of IgG positive B cells from these results support this approach to culturing B cells from cancer patients as a way to expand IgG+ memory B cells from individuals with melanoma. Next, it was determined whether the expanded memory B cells were capable of secreting IgG antibodies in sufficient quantities for further study of the humoral memory responses in patients and for examination of their reactivity and specificity to melanoma cells.

3.2.2 Production of IgG Antibodies from Human B cell Cultures

In order to confirm that it was possible to derive B cell cultures from healthy volunteers and patients with cancer, and to study the tumor reactivity of the antibodies they produce, the *ex vivo* culture and activation of patient B cells were performed similarly to those described by Traggiai and colleagues (detailed in Chapter 2, Section 2.4.2). After culturing B cells *ex vivo* for 18 days, the concentration of IgG in culture supernatants was measured by ELISA (see Chapter 2, Section 2.73). IgG concentrations were found to range from 20-45 μg/mL from B cell culture supernatants (Figure 3.4). These concentrations were comparable to the antibody amounts obtained by Traggiai et al. who found that B cells isolated

from one 35 year old patient infected with SARS coronavirus secreted IgG antibodies in the range of 3-20 μg/mL (Traggiai, Becker et al. 2004). Additionally, the production of antibodies from cells seeded at low concentrations was evaluated in order to assess the feasibility of establishing monoclonal cultures. Cultures seeded at very low cell densities, such as 1 or 2 cells per well, were found to secrete measurable antibody concentrations after 10 days in culture (Figure 3.5). In summary, prior to developing techniques to evaluate patient antibody responses to melanoma, it was crucial to verify that sufficient amounts of antibodies could be produced *ex vivo*.

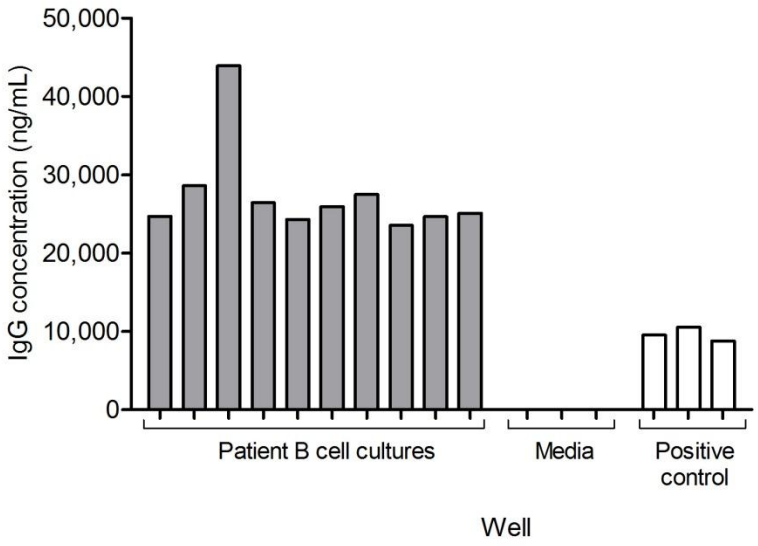


Figure 3.4 IgG production from B cell cultures derived from one patient. The amount of IgG produced from peripheral blood B cell cultures (n=10 wells) established from one patient and arising from 500 B cells per well was measured by ELISA after 18 days. Media with no B cells (n=3 wells) were used as the negative control in the ELISA. Trastuzumab, a human IgG antibody, was diluted to a concentration of 10,000 ng/mL and used the positive control antibody (n=3 wells) for the ELISA.

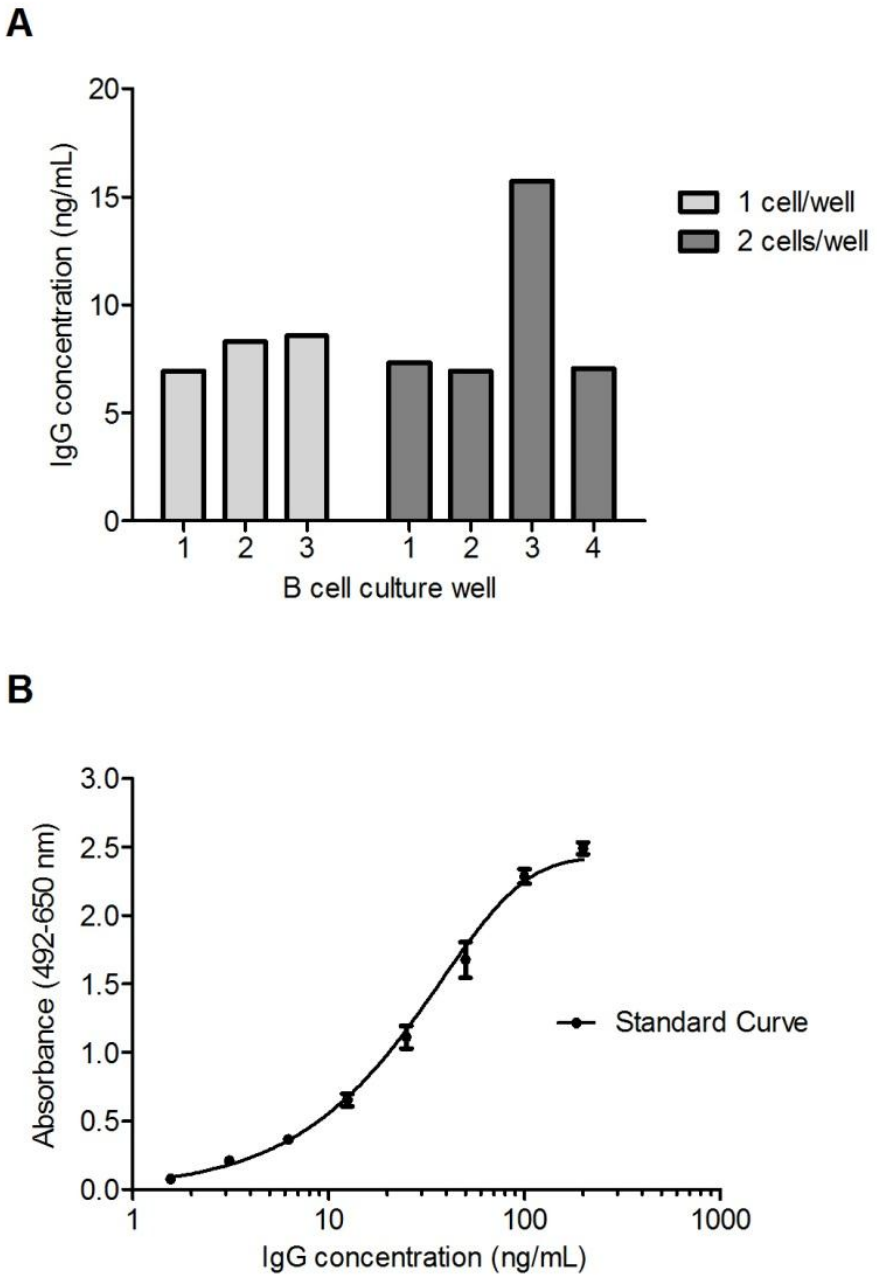


Figure 3.5 IgG production from cultures arising from 1or 2 B cells. (A) The amount of IgG secreted by patient-derived B cells diluted to 1 or 2 cells per well after 10 days in culture was measured by ELISA from cell culture supernatants. Cultures originating from 1 or 2 B cells were created by serial dilution or FACs sorting and plated in standard media along with autologous irradiated PBMCs, EBV, and CpG ODN and grown for 18 days. (B) ELISA standard curve from which results from (A) were interpolated could detect IgG at concentrations as low as 2 ng/mL.

Studies of humoral memory responses from *ex vivo* B cell cultures have been performed in the context of infectious diseases (Traggiai, Becker et al. 2004; Pinna, Corti et al. 2009), but not in cancer.[4] As a first step toward applying this methodology in melanoma, B cells derived from individuals with melanoma were compared to healthy volunteer B cells to determine whether they were able to secrete comparable levels of IgG antibodies. Since it has been reported that memory B cells from individuals with metastatic melanoma cells are hyporesponsive to activation signals as well as their reduced numbers in the peripheral blood (Carpenter, Mick et al. 2009), it was important to determine whether a reduced memory cell compartment in melanoma would impact on the presence of switched memory B cells in blood and the ability of B cells to secrete IgG antibodies in *ex vivo* cultures. These evaluations were imperative for the successful application of this approach to the study of humoral memory responses to melanoma in patients.

Similar ranges of antibody production, 1-10 μg per culture, were observed between patients (n=300 cultures derived from 5 individuals) and healthy volunteers (n=300 cultures derived from 5 individuals) (Figure 3.6). These results illustrate that while antibody production varied among individuals and culture wells, overall both volunteer groups contained switched memory B cells capable of secreting high amounts of IgG antibodies. The observed high production of antibodies from B cell cultures was essential to the process of the identification of antibody specificity and important to subsequent evaluations of reactivity to tumor cells.

[4] With the exception of the work presented within this thesis, which has been published elsewhere (Gilbert, Karagiannis et al. 2011).

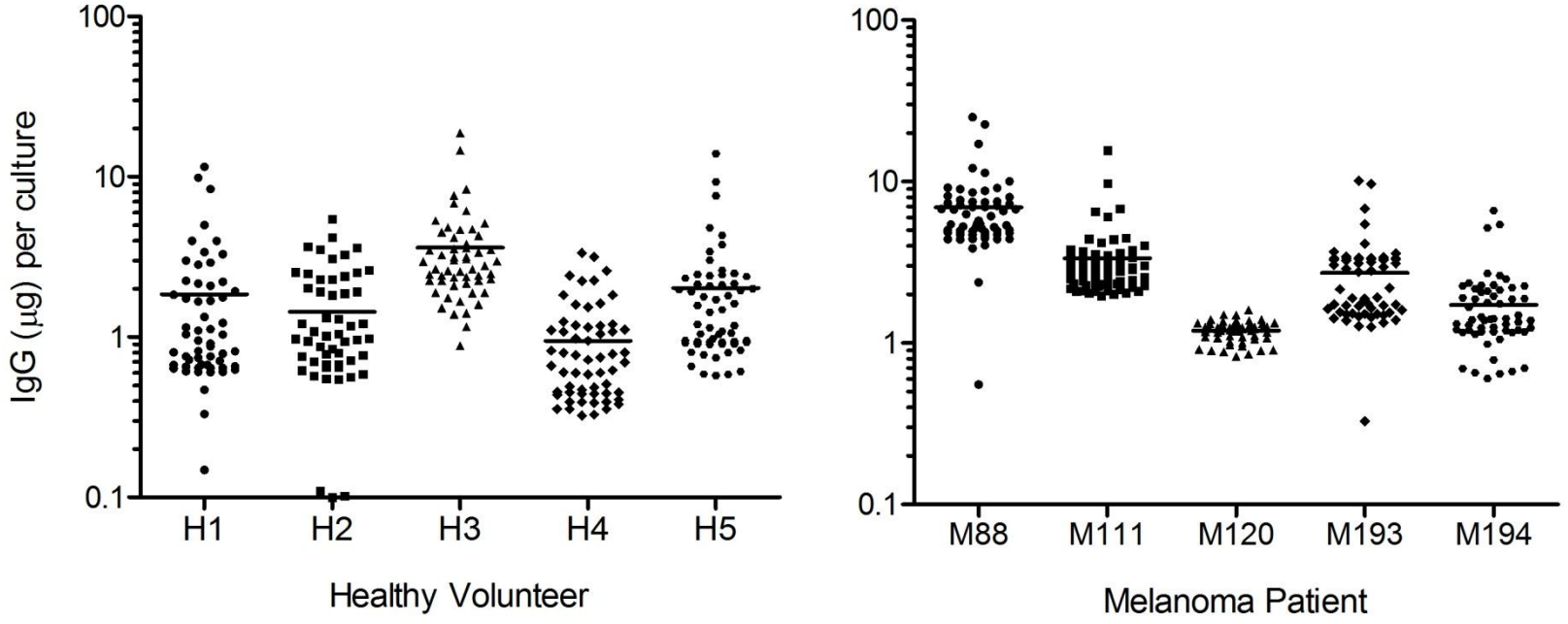


Figure 3.6 IgG production from B cell cultures established from healthy volunteers and patients. Quantification of IgG in B cell culture supernatants (each symbol represents a single B cell culture arising from 500 B cells in 200 µL of media) from healthy volunteer (n=5, left, each identifier represents a healthy volunteer) and patients (n=5, right, each identifier represents a patient) was measured by ELISA after 18 days in culture. For each healthy volunteer or patient, IgG production was evaluated for an entire culture plate (n=60 cultures) with symbols representing individual culture wells and bars representing mean IgG production for each individual.

Taken together, these results demonstrate the successful establishment of IgG antibody secreting *ex vivo* B cell cultures from the peripheral blood of cancer patients and healthy volunteers. The *ex vivo* expansion of B cells and production of IgG by human B cells reported here also support the premise that the use of a TLR9 agonist along with EBV, a method initially reported by Traggiai et al., can be utilized as a way to produce high amounts of human IgG antibodies from melanoma patients which can be further evaluated for reactivity to cell surface antigens.

3.3 Methodology to Detect Tumor-specific Antibodies from B Cell Cultures

Having found that mature antibody-secreting B cell cultures can be successfully established from patients with melanoma, the next aim of this work was to develop a methodology which would allow for the evaluation of the reactivity of these human antibodies from *ex vivo* B cell cultures to melanoma cells. As stated previously, whole cells were chosen due to the lack of recombinant melanoma surface antigens commercially available at the time of this study and the benefits of using whole cells expressing a multitude of natively-expressed cell surface antigens.

In particular, the experiments described herein were aimed at developing cell-based methods to: (1) evaluate humoral memory of patients to melanoma and (2) identify antibodies with specificity to melanoma cells.

3.3.1 Detection of Tumor-specific Antibodies by Immunocytochemistry

The detection of the specific binding of antibodies to melanoma cells was first evaluated using immunocytochemistry methods (described in Chapter 2, Section 2.5.4). Using an anti-melanoma monoclonal antibody specific for the tumor-associated antigen HMW-MAA (HMW-MAA antibody), the binding of this antibody targeting HMW-MAA could be visualized across a range (30 μg/mL to 20 ng/mL) of antibody concentrations on cytospins of A-375 melanoma cells, which are known to highly express this antigen on the cell surface (Figure 3.7, top). Additionally this HMW-MAA antibody at high antibody concentrations (30 μg/mL) was not found to bind to melanocytes (Figure 3.7, bottom left), supporting the specificity of this methodology to detect the binding of antibodies to TAAs. Lastly, the assay background was evaluated by the addition of high amounts of non-melanoma specific IgG present in healthy volunteer donor serum and was found to be negligible, as seen by the absence of antibody reactivity to A-375 melanoma cells (Figure 3.7, bottom right). This technique has the advantage of demonstrable requirement of the need for only low (20 ng/mL) amounts of antibodies for specific detection (Figure 3.7, top right). The application of this methodology in this instance yielded a qualitative method to detect tumor-reactive antibodies and required minimal assay development.

While this type of assay afforded some advantages, it also nevertheless required the production of copious amounts of slides, and the readouts were qualitative, although not easily quantitative and very low throughput. Additionally, the lack of quantitative results from this assay translated into a method which would not allow for the clear selection of cell culture wells with higher degrees of binding to melanoma cells and permit the rapid selection of B clones from thousands of cell

culture wells. Thus the application of immunocytochemistry followed by light microscopy analysis, while useful for a rapid qualitative detection of an antibody, does not provide an ideal framework to screen the specificity of antibodies to melanoma cells for thousands of patient-derived antibody cultures.

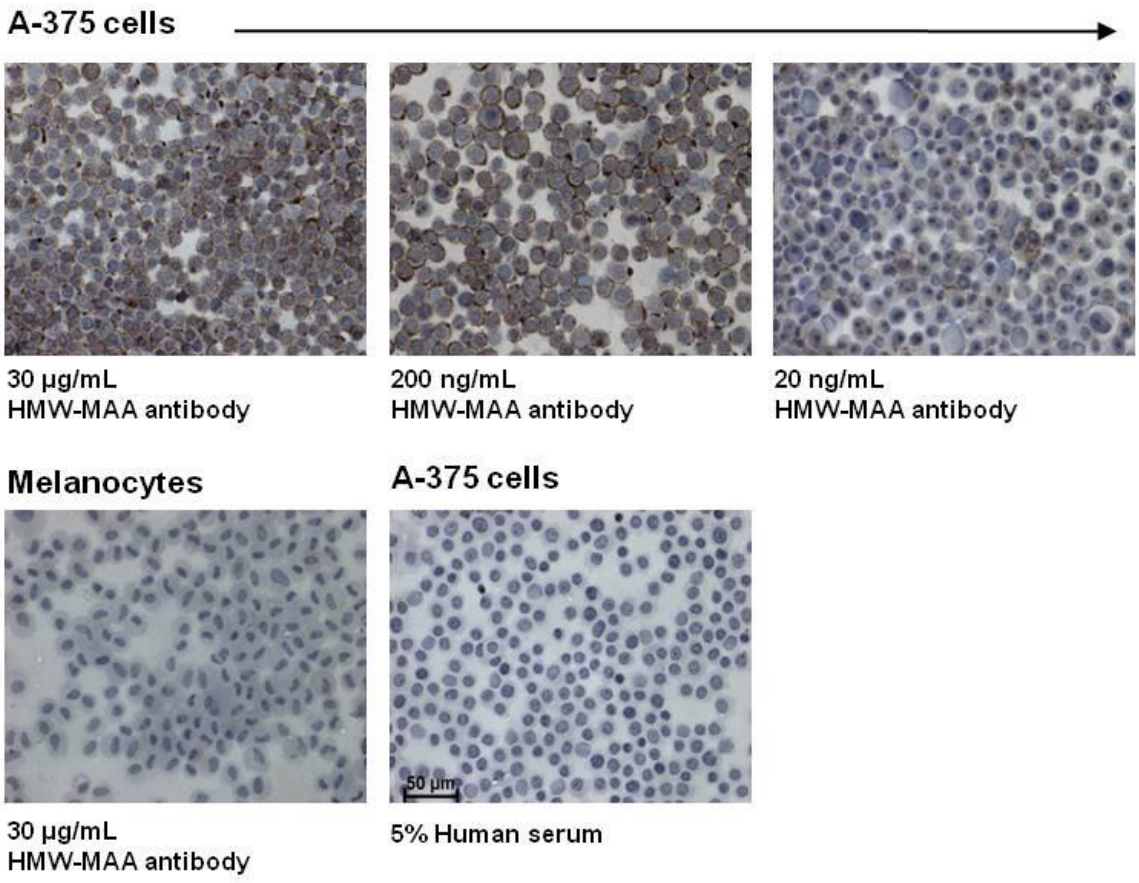


Figure 3.7 Immunocytochemical detection of a melanoma-specific antibody. Immunocytochemistry of cytospin preparations demonstrate that an antibody against the melanoma-associated antigen HMW-MAA, expressed on the surface of A-375 metastatic melanoma cells, can detect the antigen at 30 µg/mL (top left), 200 ng/mL (top middle) and 20 ng/mL (top right), while no binding to melanocytes was seen (bottom left). IgG from 5% human serum (bottom right) was not observed to bind to A-375 cells. Scale bar represents 50 µm.

3.3.2 Detection of Tumor-Specific Antibodies by Flow Cytometry

The use of flow cytometry to identify novel anti-melanoma antibodies was also explored as a potential screening tool. Unlike the previously described methods of immunocytochemistry followed by analysis with light microscopy, the fixation of cells is not required in flow cytometry. Flow cytometry presents the advantage of using live cells, negating any batch to batch variability of fixation methods and potential alteration or masking of some antigenic epitopes residing on the cell surface. Using flow cytometry, the binding of melanoma specific antibodies to melanoma cells and not melanocytes could also be seen using live cells (Figure 3.8). The specific binding of antibodies to melanoma cells could also be detected at low concentrations of antibodies, 20 ng/mL, making it comparable in sensitivity to immunocytochemistry methods using light microscopy (Figure 3.7 & Figure 3.8).

Flow cytometry, while benefiting from good sensitivity and from the use of live cells, suffered from some drawbacks as a screening tool. The primary disadvantage was the need for simultaneous maintenance of several cell lines to evaluate antibody specificity across multiple melanoma cell types as well as normal cells. In comparison to the other technique described previously, where multiple slides of the same batch of cells could be produced and stored, this would be more challenging with live cells. While more laborious in the sense of length of the assay and maintenance of cell lines, this technique could serve as a valuable method to verify antibody binding results from other methods, particularly with the regards to the effects of fixatives on antigens. In summary, while this technique was not deemed suitable for screening purposes, it could nonetheless serve as a useful tool once a melanoma-reactive antibody was identified. This flow

cytometric assay was sensitive, required small amounts of antibody, and was demonstrated to allow the identification of melanoma-reactive antibodies.

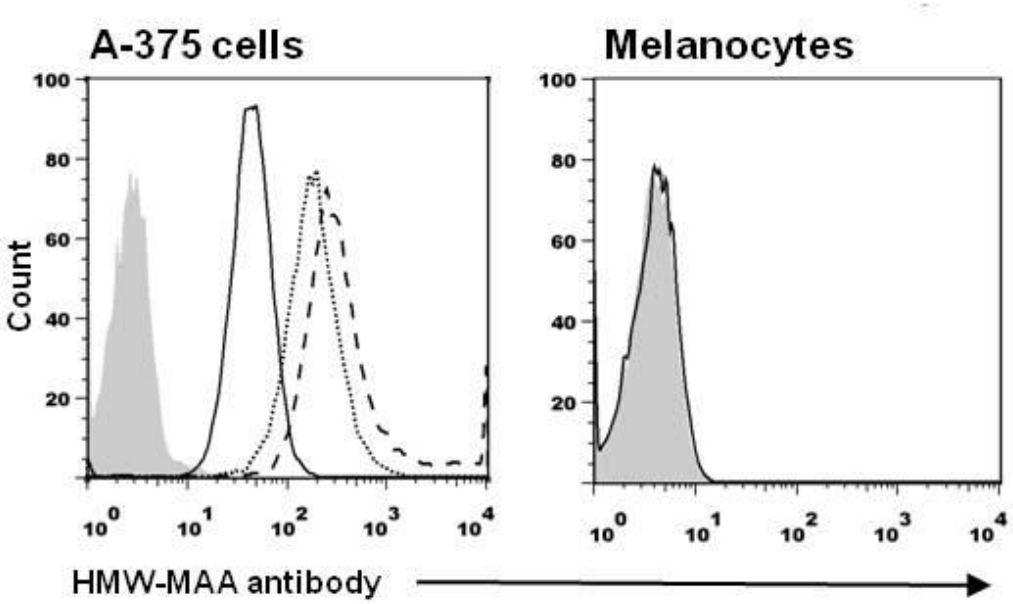


Figure 3.8 Detection of a melanoma-specific antibody by flow cytometry. Flow cytometric analysis demonstrates specific binding of the HMW-MAA antibody to HMW-MAA antigen presented on A-375 cells (left) at 30 µg/mL (dashed line), 200 ng/mL (dotted line) and 20 ng/mL (black line), but not to melanocytes (right, 30 µg/mL HMW-MAA antibody). Isotype control is shown in shaded grey.

3.3.3 Development of a Novel Cell-based ELISA to Screen for Melanoma-specific Antibodies

An ELISA was next developed and evaluated as a suitable technique to use for the screening of melanoma specific antibodies. Key requirements for this assay were that: (1) it should be suitable for use in a medium throughput manner, (2) it should permit the identification of antibodies to cell surface antigens, (3) large batches cell cultures could be made at one time and frozen, and (4) the results should be semi-quantitative. An assay fulfilling the above parameters would present as an ideal methodology to use for screening of melanoma-reactive antibodies from thousands of B cell cultures.

3.3.3.1 Assay Principle

The principle of a cell-based ELISA to detect antibodies that were bound to cell surface proteins was initially evaluated using (1) U-937 cells which express FcγRs and (2) IgG containing human sera (Anderson and Abraham 1980). A simple cell-based ELISA was constructed (Figure 3.9, left) based on a method described to measure the binding of anti-CD20 antibodies to a CD20 expressing B cell line (Hong, Presta et al. 2004). The binding of IgG antibodies present in human serum to FcγR on U-937 cells was evaluated using a colorimetric detection system. In this ELISA, the Fc-binding of IgG antibodies to cell surface FcγRs expressed on U-937 cells could be detected when compared to samples treated with buffer (culture medium) alone (Figure 3.9, right). Importantly, the detection of IgG binding (absorbance value) was decreased with decreasing amounts of IgG containing sera (Figure 3.9, right), demonstrating the potential quantitative nature of this assay.

This could later prove useful as a way to identifying cultures with the highest amount of melanoma-reactive antibodies. Results from this assay showed that the binding of IgG present in human serum to the U-937 cell line could be semi-quantitatively detected utilizing a cell-based ELISA.

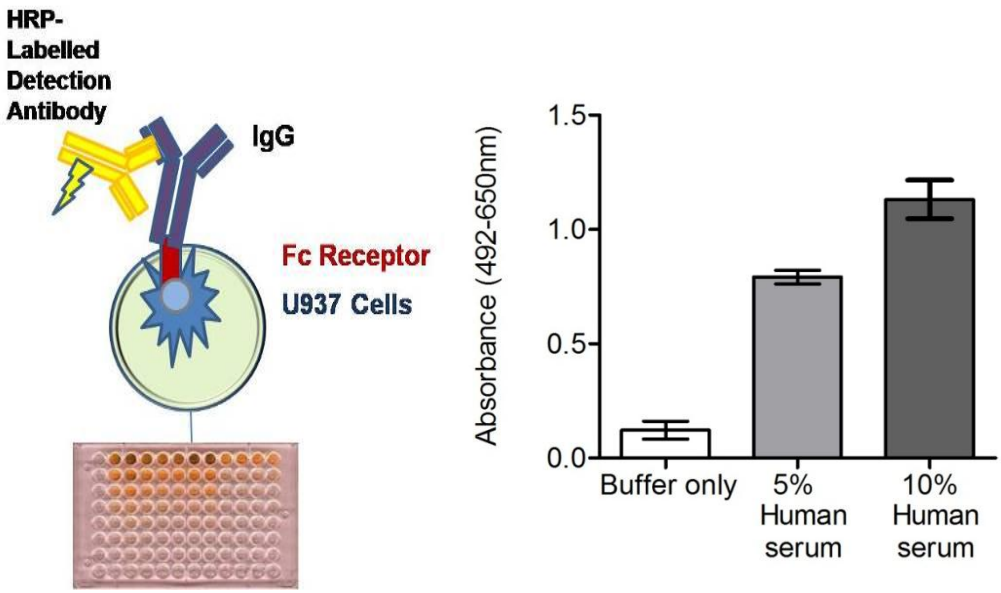


Figure 3.9 Evaluation of the proof of principle of a cell-based ELISA. The binding of IgG in human sera to the FcγRs expressed on U-937 cells was evaluated in a live cell-based ELISA. The experimental design of the assay is shown (left) along with the assay results for IgG antibodies in different concentrations of human serum compared to buffer (culture medium) only (right).

This pilot assay was performed on live cells which were kept on ice so that the results would not be confounded by effects of fixatives on antigens. But as noted previously, with the ultimate aim of developing this assay to screen thousands of patient-derived B cell culture supernatants, the use of fixed cells was highly desirable.

In designing an assay using fixed cells, the aim was to lightly fix the cells, so that the highest cytological preservation of cell surface proteins would be obtained. While a cell-based ELISA used to detect melanoma-antibodies had been previously reported (Cai and Garen 1995), the use of glutaraldehyde in this assay was not likely to meet the objective of this study, which was to measure the binding of antibodies to cell surface antigens, and prompted the development of a new assay with an alternate fixative. Glutaraldehyde is a very effective cross-linker and fixative, providing excellent preservation of cell morphology, but it may be less than ideal for use in a cell-based ELISA. The use of glutaraldehyde has been shown to result in the severe distortion and masking of cell surface molecules, thereby causing a high loss of antigenicity, artifacts and false positive recognition of some antigens residing on the surface of cells (Walker, Llull et al. 1992). With the above knowledge, alternative fixatives were explored for use in the cell-based ELISA. Two alternate preservatives commonly used for fixing cells used for cell based assays were next evaluated: methanol and formaldehyde (Walker, Llull et al. 1992; Erdile, Smith et al. 2001). The detection of antibodies against HMW-MAA expressed on melanoma cells was compared using melanoma cells fixed with either methanol or formaldehyde solution. Formaldehyde was observed to yield greater detection (absorbance value) of HMW-MAA on A-375 cells compared to methanol (Figure 3.10). The concentration of formaldehyde used in a cell-based ELISA, 0.5%, has been shown elsewhere to result in the best cell antigenicity, cell morphology, binding, consistent assay results, and to have no adverse effects on antibody-antigen binding after being stored for up to 12 months at -80˚C (Walker, Llull et al. 1992).

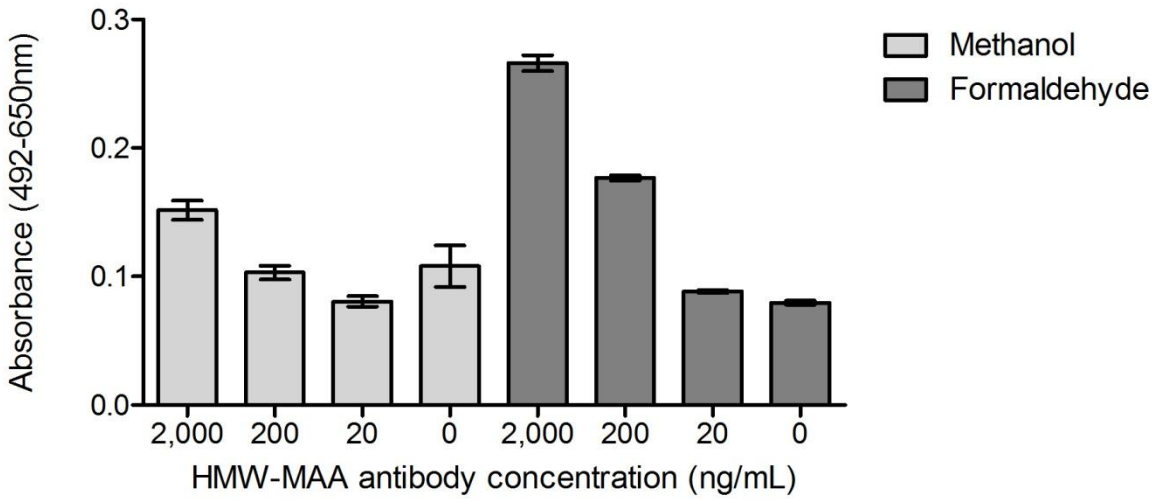


Figure 3.10 Comparison of different fixatives for use in the cell-based ELISA. During early assay development cell fixatives were evaluated due to high background using live cells. The detection of HMW-MAA expressed on the cell surface of A-375 melanoma cells was evaluated by a cell-based ELISA for cells treated with either methanol or formaldehyde, diluted to 0.5% in HBSS. Each condition was tested in triplicate and error bars represent one standard deviation (SD).

After formaldehyde was chosen as the cell fixative, the effect of plate washing on cell adherence was investigated. Cells were seeded at a density of 3×10^5 cells per well on 96 well plates and grown until confluent. Plates were then washed 18 times to replicate each step of washing in the ELISA and adherence was monitored by light microscopy. A representative image shows the high adherence of cells after multiple plate washing steps (Figure 3.11). The lack of cell loss is important, in order to ensure an adequate and uniform number of cells for each assay.

Figure 3.11 Evaluation of washing steps on cell adherence in the cell-based ELISA. Adherence of A-375 melanoma cells to wells was monitored by light microscopy. A-375 melanoma cells were seeded on 96 well plates and grown until confluent. Cells were then fixed with 0.5% formaldehyde in HBSS, wrapped in aluminum foil, and stored at -80ºC until day of assay. After plates were thawed at room temperature for 30 minutes, wells were washed 18 times to replicate washing steps in the assay and the adherence of cells was monitored by light microscopy. Representative image shown at 80x magnification.

These assessments of fixatives and cell adherence were the initial steps in developing a cell-based ELISA, a tool to detect antibodies recognizing melanoma antigens. Using cells highly adherent to 96 well plates and fixed with 0.5% formaldehyde, a cell-based ELISA was created and incubation times and reagents were next optimized to create the highest antibody-antigen detection system.

3.3.3.2 Assay Optimization

Several parameters were evaluated in the development of a cell-based ELISA for the detection of melanoma-reactive antibodies. This optimization was performed using the A-375 melanoma cell line, which highly expresses the HMW-MAA, and an antibody targeting this antigen. Optimal conditions such as incubation time, detection time and antibody concentrations were defined as the shortest incubation time, or least amount of reagent, or fewest additives, where the quality of the results was not decreased.

One such parameter evaluated was the optimal incubation time of antibody containing cell culture supernatants to adherent tumor cells. Using the HMW-MAA antibody diluted in B cell culture media, antibody incubation time was evaluated at 30 minute intervals: 60, 90 and 120 minutes, along with multiple decreasing concentrations of HMW-MAA antibody: 250, 125, 62.5, and 31 ng/mL. It was found that absorbance values for the 90 and 120 minute incubation periods were similar, while the 60 minute incubation had a lower absorbance value (Figure 3.12A, left). For these conditions, there was not a large reduction in the assay background (buffer only condition) among the three incubation times, suggesting that any non-specific binding of bovine proteins present in the culture media was not increased over time (Figure 3.12A, right). Detection of the antibody at concentrations as low as 31 ng/mL was observed with an incubation time as low as 60 minutes. However, the incubation time of 90 minutes was selected due to increased absorbance values at this time point, while an additional incubation of 30 minutes (120 minutes total incubation) did not provide significant increases in absorbance values.

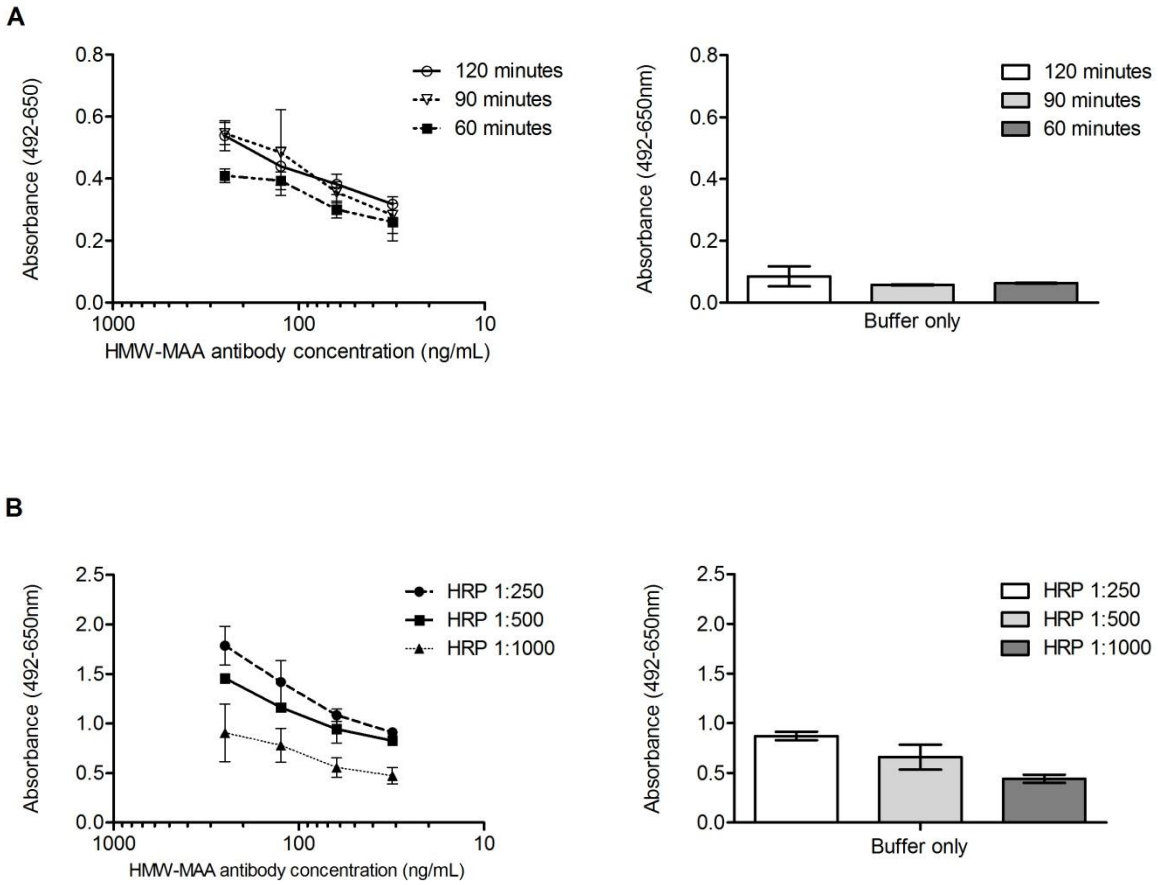


Figure 3.12 Evaluation of selected parameters for the development of a fixed cell-based ELISA. A cell-based ELISA was optimized to detect melanoma-reactive antibodies using the A-375 melanoma cell line and a HMW-MAA antibody. Primary incubation time was evaluated for antibodies over a range of concentrations and buffer to optimize antibody detection (A, left) and non-specific binding (A, right). The secondary antibody was also evaluated at multiple titrations (B, left) to achieve optimal antibody detection and reduced background (B, right).

Another key parameter evaluated in the development of the cell-based ELISA was the concentration of the secondary antibody, a $F(ab')_2$ antibody fragment specific for the IgG Fc region, which was used to detect the binding of melanoma-reactive antibodies to tumor cells. In this optimization step, the criteria were set to select the condition with highest antibody detection (absorbance value) accompanied by the lowest background. The detection antibody was tested at three dilutions: 1:250, 1:500 and 1:1000, with 1:250 being the highest amount of antibody that could be used in a reasonably cost-effective manner. The highest detection of the anti-HMW-MAA antibody along with highest background was observed with the highest amount of antibody, at a 1:250 dilution (Figure 3.12B). Since a higher amount of background was also observed with the highest amount of detection antibody, the antibody diluted 1:500 was selected for the assay since this concentration had reduced background while still maintaining adequate detection of melanoma-reactive antibodies to melanoma cells.

3.3.3.3 Verification of Assay Sensitivity and Specificity

After setting up the final parameters of cell-based ELISA, the assay was further evaluated for the following two key factors: assay sensitivity and assay specificity. First, it was crucial to determine that this assay could specifically detect the binding of antibodies to antigens on tumor cells and not just the binding of non-specific antibodies to 96 well plates or the non-specific binding of antibodies to cells. Secondly, it was important to ensure that the assay was sensitive enough to adequately detect antibodies present in patient B cell culture supernatants.

Assay sensitivity was assessed by comparing the binding of the melanoma-specific HMW-MAA antibody diluted to a range of concentrations to A-375 melanoma cells. The binding of the melanoma-specific antibody to antigens expressed on the surface of melanoma cells could distinctly be detected and differentiated at antibody concentrations as low as 10 ng/mL by the cell-based ELISA (Figure 3.13). Based on the concentration of IgG measured from cultures arising from one or two B cells 10 days after their establishment (Figure 3.5), an assay with a lower limit of sensitivity of 10 ng/mL would likely detect melanoma-reactive antibodies present in patient B cell culture supernatants. Thus, this method could theoretically detect melanoma-reactive antibodies secreted from a single B cell clone.

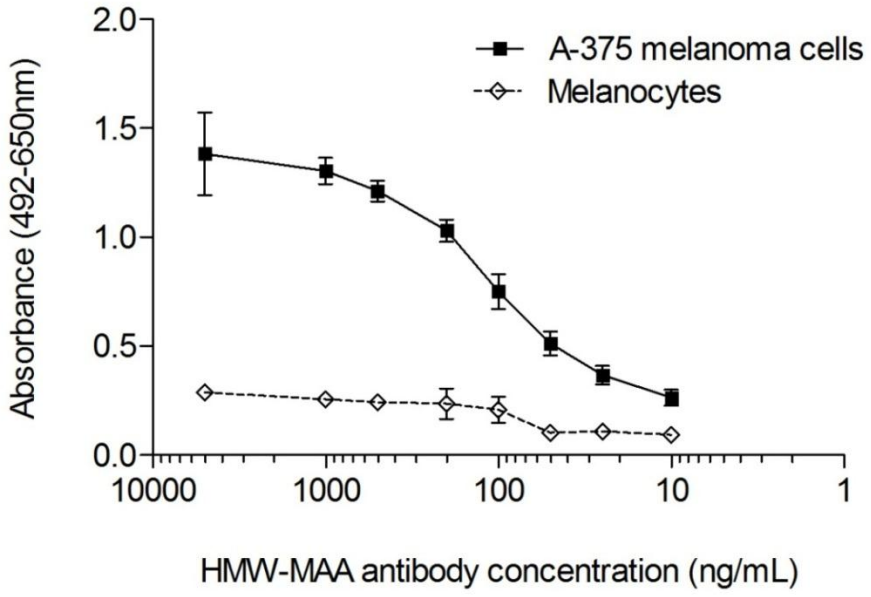


Figure 3.13 Detection of melanoma-specific antibodies to melanoma cells and not to melanocytes by a cell-based ELISA. Detection of the HMW-MAA antibody to A-375 melanoma cells over a range of antibody concentrations was compared to melanocytes. The HMW-MAA antibody was tested in triplicates for each concentration and error bars represent one SD.

The ability of the cell-based ELISA to detect antibodies bound to cell surface antigens expressed on cancer cells was also verified using a commercially available antibody. The detection of the binding of Trastuzumab, a monoclonal antibody against the tumor associated antigen Her2/neu (whose proto-oncogene is amplified/overexpressed in 25-30% of primary breast cancers) to breast cancer cells was evaluated using the cell-based ELISA (Slamon, Godolphin et al. 1989). The assay was performed exactly as previously described (Chapter 2, Section 2.7.5), with the exception that the breast cancer cell line SK-BR-3 rather than melanoma cells or melanocytes was used to coat the 96 well plates. Performing this assay, it was possible to specifically detect Trastuzumab binding to breast cancer cells (Figure 3.14), supporting the robustness of this assay to detect tumor-specific antibodies as well as the potential application of this methodology to study host B cell responses to other cancers.

The specific detection of antigen-antibody binding was also evaluated for melanoma cells. Comparing cell culture media containing no human antibodies, non-specific human IgG and an antibody targeting melanoma cells, the specific detection of antibodies against melanoma cell surface proteins could be measured by this ELISA (Figure 3.15).

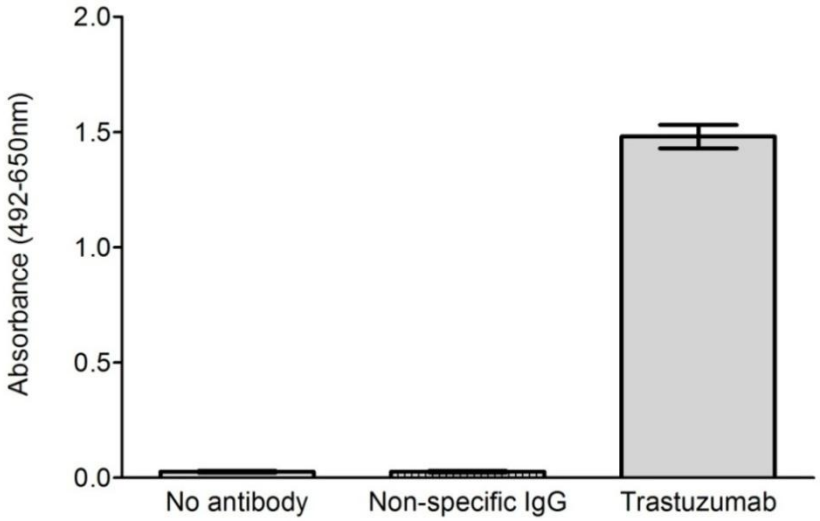


Figure 3.14 Utilizing the cell-based ELISA to detect the binding of a commercially antibody therapeutic to cancer cells. The binding of Trastuzumab to SK-BR-3 breast cells compared to an IgG isotype control or media only (no antibody) using the cell-based ELISA. Conditions were tested in triplicate and error bars represent one SD.

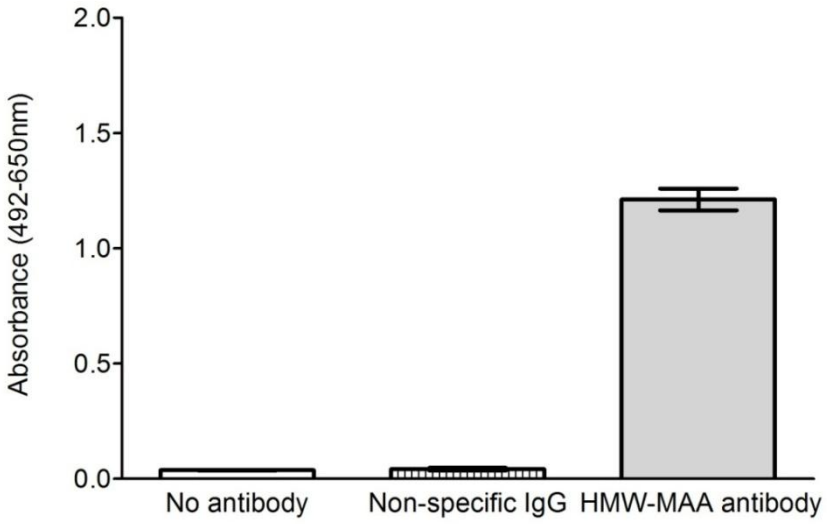


Figure 3.15 The specific detection of melanoma antibodies against melanoma cell surface antigens by a cell-based ELISA. Melanoma-specific antibody, HMW-MAA antibody, binding to A-375 melanoma cells was compared to an IgG isotype control in the absence of antibody. Conditions were tested in triplicate and error bars represent one SD.

By comparing the binding of either the anti-HMW-MAA antibody or Trastuzumab to tumor cells to that of media containing bovine IgG or to the non-specific IgG control (which could represent irrelevant IgG antibodies secreted from the human B cells), non-tumor antigen-specific antibodies were not observed to become immobilized on the plates themselves or seen to become non-specifically bound to proteins on tumor cells. If this were to occur high absorbance values would have been detected for the non-specific IgG control or media only. Rather, the assay specifically detected the binding of tumor-specific antibodies to tumor cells (Figure 3.14 & Figure 3.15). Furthermore, upon comparing the addition of HMW-MAA antibody to plates coated with A-375 melanoma cells to those coated with melanocytes, the specific binding of this antibody to melanoma cells, and not to normal cells such as melanocytes, was observed (Figure 3.13), verifying the specificity of the assay.

3.3.3.4 Selection of Positive and Negative Controls

Integral to screening for melanoma-reactive antibodies was the choice of appropriate controls for the ELISA. Controls were necessary not only to monitor the performance of the assay, but also to compare results across assays, cell lines and individuals. For the negative control, a human (non-specific) IgG antibody was selected. This negative control ensured that assays with high amounts of non-specific background would be discounted from analyses. The HMW-MAA antibody, used in the previous sections to develop the assay, was next evaluated for use as a positive control in the cell-based ELISA. The binding of this antibody to 5 melanoma cell lines was evaluated by FACS, and expression of HMW-MAA was

detected on the 5 melanoma cell lines evaluated (Figure 3.16, top). Expression (level of fluorescent intensity above isotype control) of HMW-MAA varied among melanoma cell lines, with a 9-fold difference between A-375 and WM-115 cells and 60-fold difference between A-375 and SK-MEL-28 cells (Figure 3.16, top). The variable antigen expression of the HMW-MAA among melanoma cell lines would make it challenging to set general cut-off points by which to select positive cultures based on the positive control absorbance values. This is because expression levels could be log scale values apart among available panels of cell lines (Figure 3.16, top). Additionally, the lack of expression of this antigen on non-malignant cells such as melanocytes (Figure 3.16, top) would require a separate positive control antibody for such cells used in the ELISA. Since screening would not only require the comparison of antibody reactivity to melanoma cells but also to melanocytes, it was preferable to choose an antigen expressed more uniformly on both melanoma cells and melanocytes.

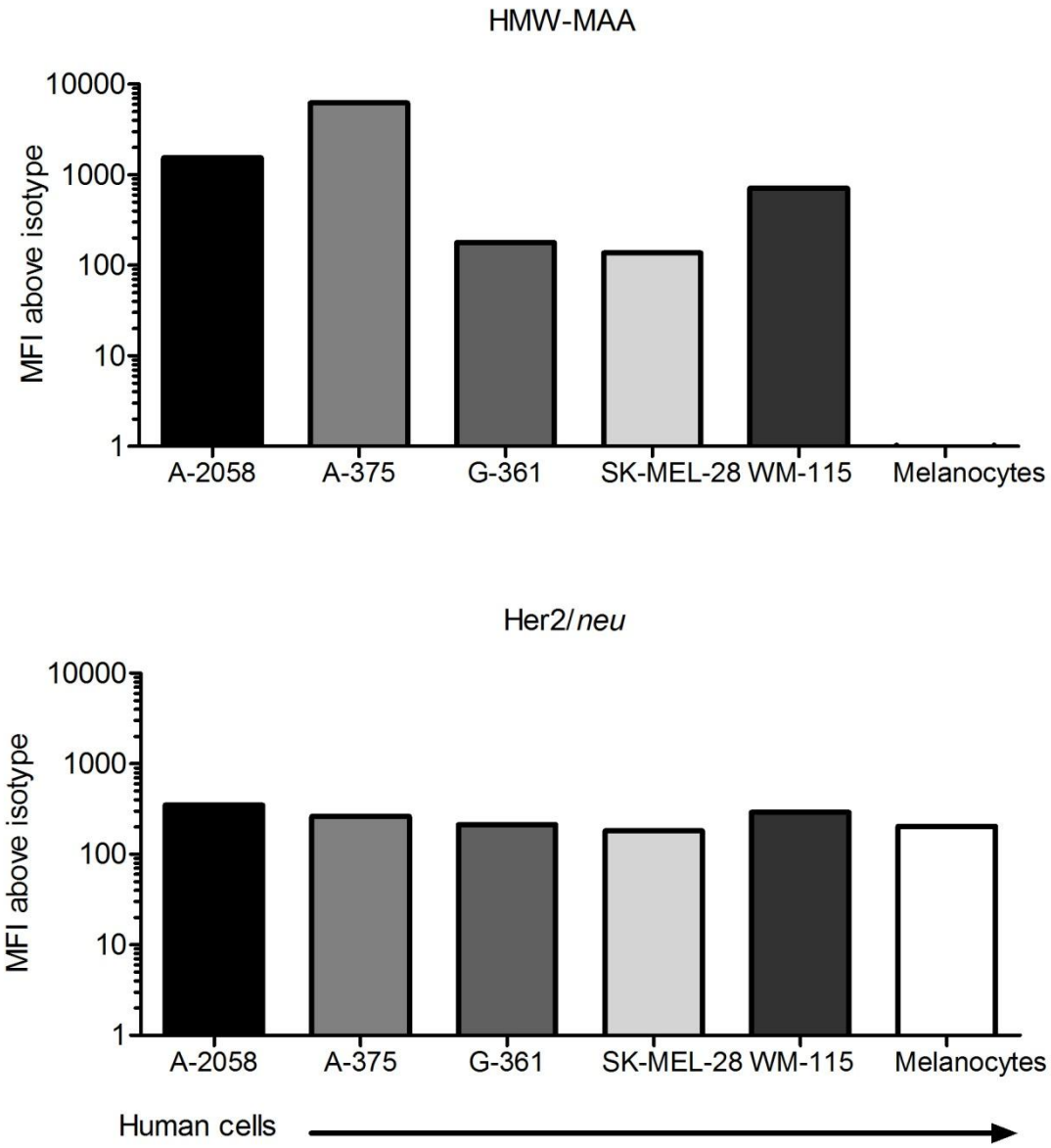


Figure 3.16 Expression of two tumor cell surface antigens on melanoma cell lines and melanocytes. Tumor cell surface antigen expression on five melanoma cell lines and primary melanocytes was evaluated by flow cytometry. HMW-MAA (top) was detected with the 225.28S chimeric-HMW-MAA IgG antibody and Her2/neu (bottom) was detected with Trastuzumab.

In summary, a specific and adequately sensitive cell-based ELISA with appropriate positive and negative controls was developed for the purpose of identifying novel patient derived melanoma-reactive antibodies. Exploration of other methodologies such as immunocytochemistry followed by light microscopy evaluations and flow cytometric methods were useful in assessing the specificity of an antibody and provide additional tools to examine the effects of fixation on antigenicity, but also serve as confirmation of the ELISA results, since similar results were seen for the detection of the anti-HMW-MAA antibody across these three different methodologies. Additionally, these assays can be adapted to study many different adherent malignant and non-malignant cell lines, as seen by the example of the detection of Trastuzumab binding to SK-BR-3 breast cancer cells, in order to screen for tumor-reactive antibodies from human B cell cultures established from patients with other cancers.

3.4 Proof of Principle: Reactivity of Antibodies Isolated from Individuals to Melanoma Cells

Having developed an adequate screening assay to detect anti-tumor antibodies from human B cell culture supernatants, the next stage was to investigate whether any melanoma-reactive human IgG antibodies could be identified from *ex vivo* peripheral blood B cell cultures. To test whether melanoma-reactive IgG antibodies could be readily detected, peripheral blood B cells from a small cohort of patients with melanoma were studied and compared to B cells derived from the blood of healthy volunteers. This section details the process by which patient-derived antibodies produced in primary B cell cultures were screened for reactivity to cells.

3.4.1 Process for Screening Human IgG Antibodies

Once B cell cultures were established from patients, cultures were grown for 18 days and antibodies secreted in these primary cell culture supernatants were screened against melanoma cell lines or non-malignant cells using the cell-based ELISA described in the previous section (method detailed in Chapter 2, Section 2.7.5) in order to identify melanoma-reactive B cell cultures. The steps in this process are highlighted in Figure 3.17.

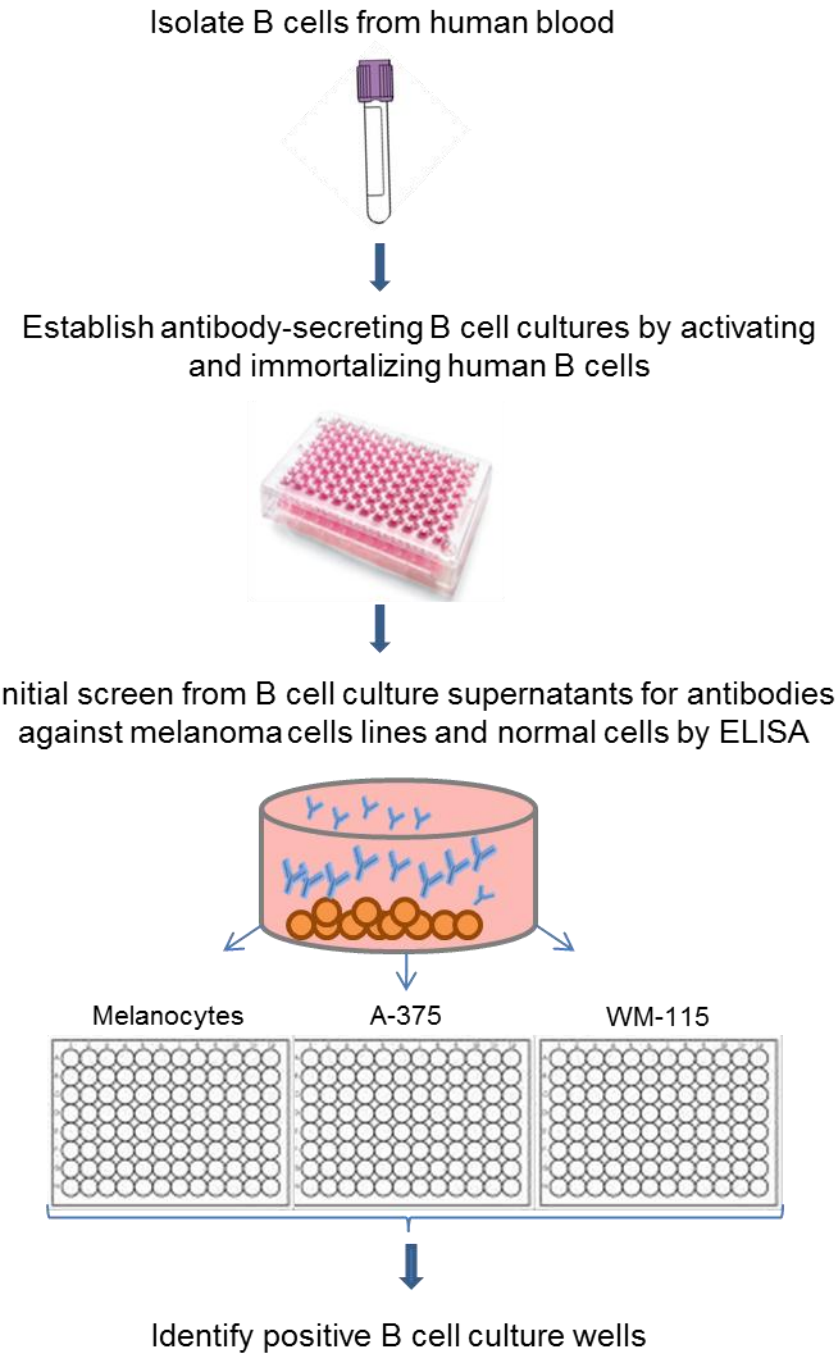


Figure 3.17 Process for identifying melanoma-reactive antibodies from human B cells. Schematic of process used to identify melanoma-reactive antibodies from patients with melanoma. Freshly isolated B cells from peripheral blood were activated with CpG ODN and transformed with EBV and cultured along with irradiated autologous PBMCs as feeder cells for 18 days. After 18 days in culture, cell culture supernatants were removed and tested using the cell-based ELISA for reactivity to melanoma cells (A-375 or WM-115) or to normal cells such as melanocytes. The cell-based ELISA results were used to initially select positive melanoma-reactive cultures for further expansion and analyses.

3.4.2 Criteria for the Assessment of Antibody Reactivity to Tumor Cells

Utilizing the cell-based ELISA for screening, the next criteria explored were concerned with the selection of melanoma-reactive B cell cultures. While others have defined positive reactivity to be the mean absorbance value of the negative control antibodies plus 3 times the SD of the mean (Kirkwood and Robinson 1990), more stringent criteria were set in this study, which allowed for the selection of cultures with the highest reactivity to melanoma cells for further sub-cloning.[5] The use of stringent criteria ensured that results would be less likely to reflect false positive values obtained by ELISA and more likely lead to the selection of antibody clones with reactivity to melanoma cells. A cut-off point was used for each assay, to select B cell cultures by choosing wells with absorbance values measured to be at or above 75% of the absorbance value of the positive control (in this instance Trastuzumab) to melanoma cells (Figure 3.18). In the example presented in Figure 3.18 below, two B cell cultures had absorbance values which produced optical density values of at least 75% of the positive control; cultures would be selected and expanded for further reactivity evaluations.

[5] A comparison of these criteria used herein to those used by others is presented in the Appendix.

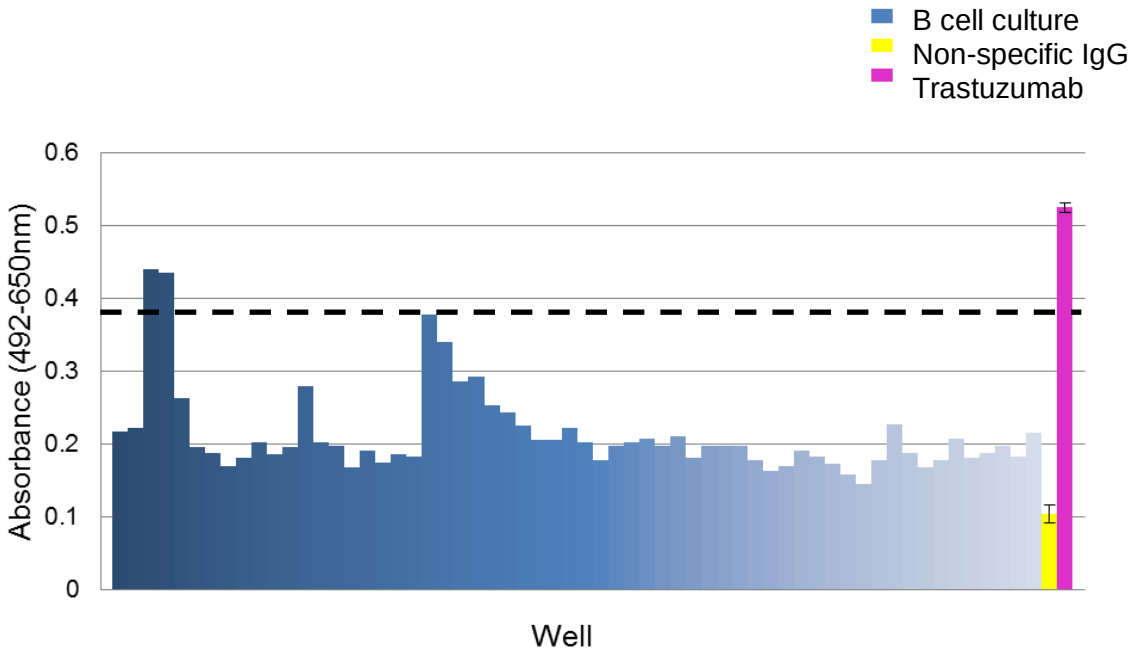


Figure 3.18 An example of cell-based ELISA results for patient B cell culture supernatants along with negative and positive assay controls. Data from the cell-based ELISA measuring the reactivity of antibodies in B cell culture supernatants (blue) from one patient (M133) B cell culture plate to WM-115 melanoma cells. Each bar represents reactivity of a supernatant removed from a single B cell culture. Non-specific human IgG antibody (yellow) was used as the negative control and Trastuzumab (pink) served as the positive control. Both controls were tested in triplicates. The dashed line represents the 75% absorbance value of the positive control, a cut-off used to choose clones for further evaluations.

3.4.3 Initial Assessments of the Detection of Melanoma-reactive Antibodies from Human B cell Cultures

The possibility of the identification of melanoma-reactive antibodies from *ex vivo* human B cell cultures from a patient with melanoma (M120) was explored using the newly established cell-based ELISA. In these experiments, the binding of antibodies produced in these cultures was studied against two separate melanoma cell lines as well as against melanocytes. Analysis of the reactivity of these B cell cultures (n=120) revealed that antibodies from this patient bound to melanoma cells (A-375 metastatic melanoma and WM-115 primary melanoma cells) and overall had less reactivity to melanocytes (Figure 3.19). The patient evaluated in this example was arbitrarily selected and at the time of B cell isolation was classified as having Stage II melanoma. These patient cultures along with those from another patient (Figure 3.18) served as examples of the feasibility of this approach to identify melanoma-reactive antibodies from patients. While findings of melanoma-reactive IgG antibodies from patient B cells have been documented, these findings have been restricted to only a few antibodies; furthermore, some were of the IgM class from individuals with unusual clinical progressions, such as autoimmune responses, such as Vitiligo followed by disease-free survival of over 6 years (Kirkwood and Robinson 1990). Such findings may provide insights into mechanisms of relapse-free survival, and suggest that humoral responses may form a component of these responses in selected patients. These individual cases notwithstanding, the objective here was to assess how widespread a host B cell response to melanoma was across individuals, and how applicable this new cell-based ELISA would be towards identifying monoclonal antibodies recognizing melanoma. These promising findings from the very first two patients studied

supported this approach as a potentially useful method to evaluate and discover melanoma-specific antibodies from patients.

A limitation of these initial assessments related to the rigor of evaluation of these antibody cultures using a cell-based ELISA, being restricted to the examination of up to three or four cell lines (Figure 3.19) at any one time. Only 3 assays, one for each cell line, could reasonably be performed from B cell cultures at a given time due to the volume of available cell supernatant from each culture. This limitation restricted the level of reactivity one could examine against different cells, and the ability to test each culture supernatant in triplicate. In the future, such a limitation could be overcome by further refinement of the cell-based ELISA such as the use of 384 well plates, which would require less volume of supernatant, or by increasing the level of sensitivity of the assay with the use of a fluorescence detection system rather than the present colorimetric one, so that less supernatant would be required per assay. Alternately, other methodologies could be utilized as tools to study humoral immune responses and identify novel antibodies and antigens beyond the cell-based ELISA, such as the use of protein microarrays. Protein microarrays have been described to have the potential to detect the reactivities of antibodies against possibly thousands of proteins and have recently be used to evaluate humoral immune responses in ovarian, pancreatic and colorectal cancers (Gnjatic, Ritter et al. 2010; Babel, Barderas et al. 2009). While improvements to the cell-based ELISA could allow for further evaluation of additional cell lines or the additional use of other tools such as arrays, even one ELISA coated with one melanoma cell line could possibly provide sufficient information to select melanoma-reactive cultures which could subsequently be further expanded and evaluated for reactivity to more melanoma cells along with normal skin cells.

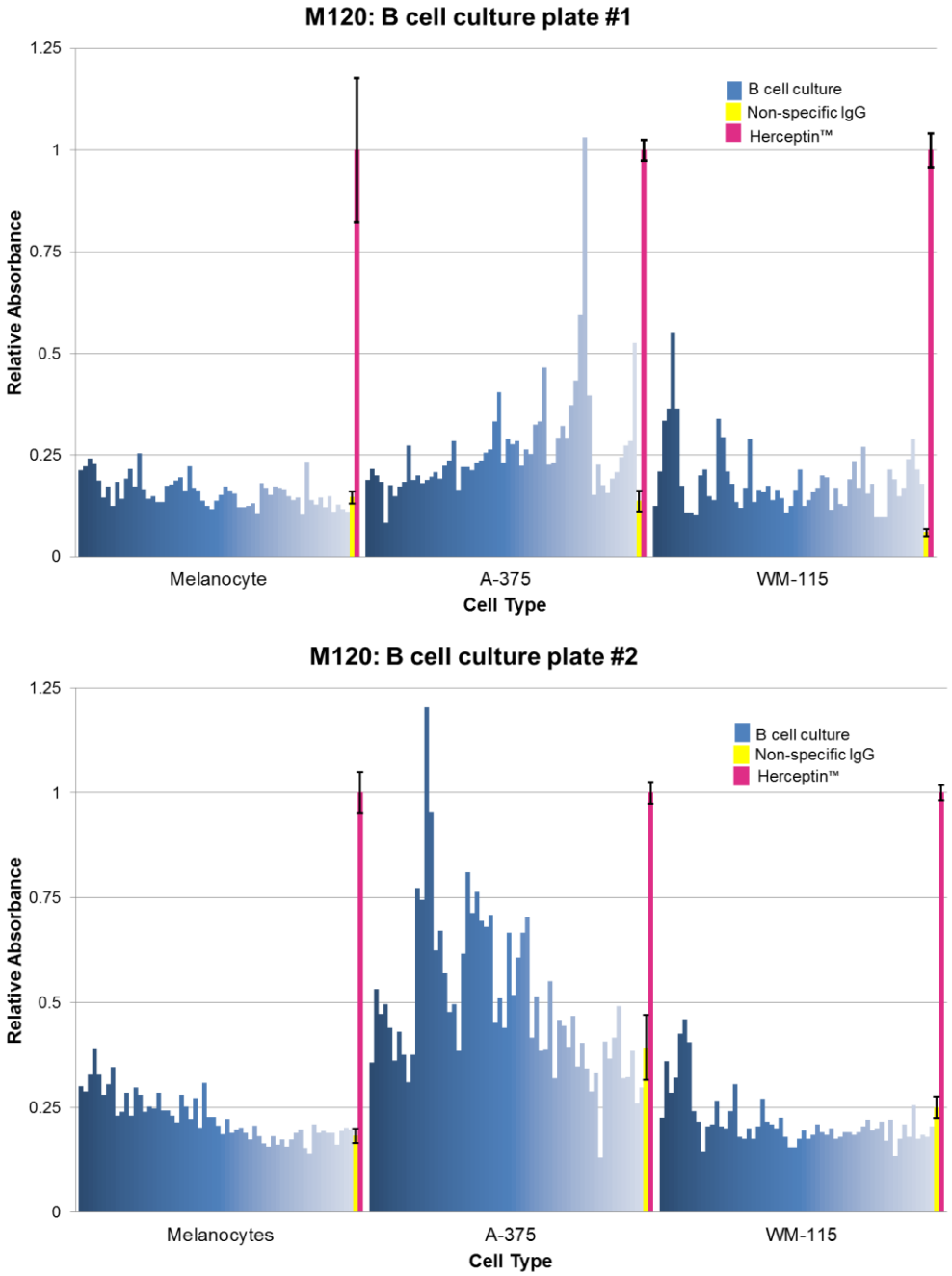


Figure 3.19 Assessing the reactivity of antibodies in patient B cell culture supernatants to multiple cell types by ELISA. The reactivity of antibodies in B cell culture supernatants (blue, n=120) from patient M120 was assessed using the cell-based ELISA. Antibody reactivity was evaluated against three cell types by ELISA: primary melanocytes, the metastatic melanoma cell line A-375, and the primary melanoma cell line WM-115. Absorbance values were normalized across assays relative to the positive control, Trastuzumab (pink), which was tested in triplicate. Non-specific IgG (yellow) was used as a negative control and also tested in triplicate. Error bars represent one SD.

Next, the reactivity of antibodies to melanoma cells was further evaluated from B cell cultures established from a cohort of six patients and 6 healthy volunteers. Results from the cell-based ELISA show that antibody cultures (n=360) from patients (n=6) had overall higher antibody reactivity to both primary and metastatic melanoma cells, compared to antibody cultures derived from healthy volunteers (n=6 individuals; 360 cultures), which were tested against the same cell lines (Figure 3.20). Healthy volunteer B cell cultures all had absorbance values below the 0.75 relative absorbance cut-off point for the cell-based ELISA using A-375 and WM-115 melanoma cells (Figure 3.20). Strikingly, it was observed that melanoma-reactive antibodies were detected in more than one individual with melanoma against each cell line (Figure 3.20). The observation that anti-tumor antibody responses (cultures above the cut-off point) were observed with patient B cell cultures, but not with healthy volunteer B cells, further supports the capacity of this cell-based ELISA to specifically detect antibody responses to malignancy.

Upon examination of the reactivity of B cell culture supernatants to the A-375 and WM-115 melanoma cell lines, all six patients had at least one culture with reactivity to the A-375 cell line, while three patients had at least one culture reactive to the WM-115 cell line (Figure 3.21). In these preliminary investigations, it was also observed that a few cultures found in two out of the six patients had reactivity to both melanoma cell lines evaluated (Figure 3.21). This may be due to antibodies arising against an antigen expressed on both cell lines in these patients. These cultures are of great interest because they bind to two different melanoma cell lines, one which was derived from a primary melanoma from one individual (WM-115) and the other from a metastatic melanoma lesion from another individual (A-375). Such antibodies may be against commonly expressed antigens

in melanoma and, in principle, these may be of potential diagnostic or therapeutic interest.

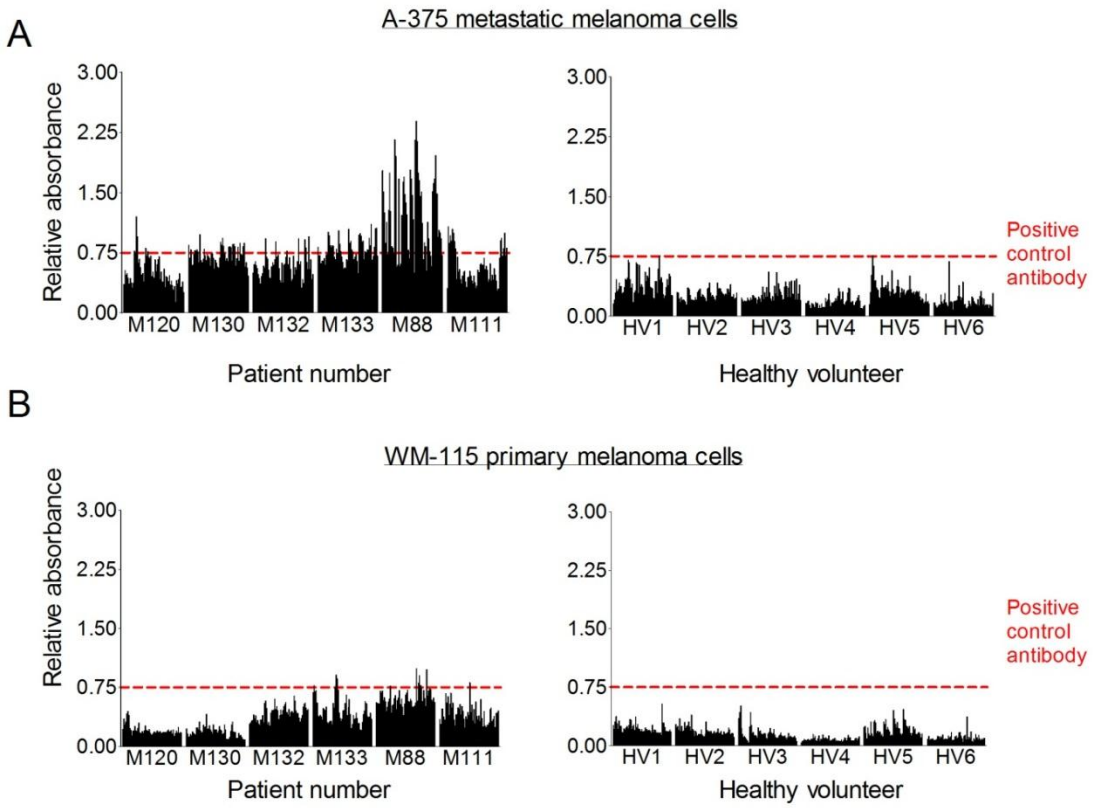


Figure 3.20 Initial assessment of the reactivity of antibodies derived from melanoma patients and healthy volunteers to melanoma cells. Antibodies in B cell culture supernatants derived from healthy volunteers (n=6) and melanoma patients (n=6) were evaluated for reactivity to A-375 metastatic melanoma cells (A) and WM-115 primary melanoma cells (B). Culture supernatants from both healthy volunteers (n=360 cultures) and patients (n=360 cultures) were evaluated for reactivity to melanoma cells using a cell-based ELISA. Absorbance values across assays were normalized based on a positive control antibody assigned a relative absorbance of 1. The relative absorbance unit of 0.75 was set as a guide for choosing clones for further selection.

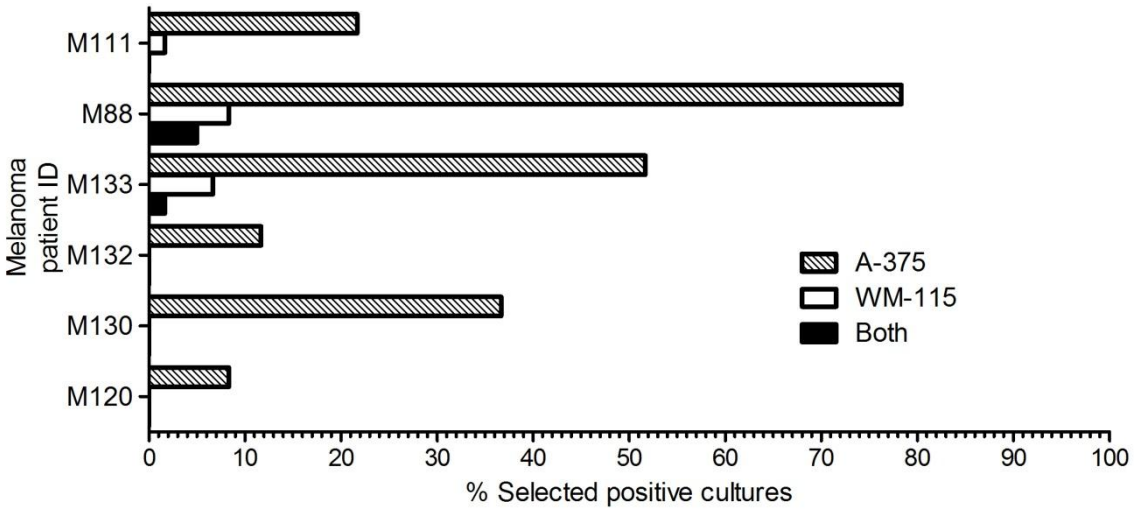


Figure 3.21 Estimation of positive melanoma-reactive antibody cultures from 6 patients. The number of positive cultures from 6 patients evaluated against two cell lines: A-375 and WM-115 using the cell based ELISA (see Figure 3.20). Cultures were counted as positive if the absorbance value was at least 75% of the control antibody. Cultures with wells selected as positive for both cell lines are shown in black bars.

The finding that each of the 6 individuals studied had at least one tumor cell-reactive antibody culture (Figure 3.21), suggests that a memory B cell response to melanoma is present in many individuals with cancer. Taken together, these results suggest that a common host B cell response to melanoma exists among this patient cohort and the cell-based ELISA was a proficient tool to identify melanoma cell-reactive cultures.

3.5 Conclusions

This chapter demonstrated that it was possible to establish *ex vivo* IgG antibody-secreting B cell cultures from the peripheral blood of individuals with melanoma, as well as from healthy volunteers, and presents a cell-based ELISA method to rapidly identify novel tumor cell-reactive antibodies from clinical specimens.

Patient-derived B cell cultures were observed to produce antibodies that recognize melanoma cells derived from primary and metastatic tissues. This reactivity to melanoma cells was not observed in healthy volunteers who have not had exposure to melanoma, supporting the premise that there are specific mature memory B cell responses to cancer. Further study showed that some B cell cultures have antibodies that bind to melanoma cells, but do not recognize normal melanocytes. These results show great promise for the identification of novel melanoma antibodies from patient specimens. Applications of these methods to evaluate humoral responses to melanoma are presented in Chapter 4, and efforts to discover monoclonal antibodies from patients are presented in Chapter 5.

The methodology described herein includes the establishment of antibody-secreting B cell cultures, a screening assay, and a process to identify cultures with melanoma-reactive antibodies. This work potentially has multiple clinical applications, including the discovery of new diagnostic and therapeutic antibodies which could also reveal novel cell surface antigens. Melanoma-reactive antibodies isolated from patients could be co-immunoprecipitated with antigen, which could lead to the identification of novel antigens by mass spectrometry. Such an approach has also been employed using phage display libraries to construct IgG1 antibodies following antigen identification, and it was shown that some of these

antibodies were able to mediate ADCC *in vitro* (Kurosawa, Akahori et al. 2008). The use of human antibodies rather than phage display library-derived clones would negate the need to construct IgG antibodies, and affinity-matured antibodies could be immediately tested for their ability to mediate ADCC. Also, these novel antigens could be used as imaging agents, biomarkers, or diagnostic tools.

Furthermore, the detection of melanoma-reactive antibodies from patients but not from healthy volunteers using the cell-based ELISA points to the potential diagnostic utility of this method. With further stringent optimization, this ELISA could be employed as a non-invasive diagnostic tool to identify the presence of a humoral response to melanoma in individuals. This assay could also benefit from technical improvements, including the use of multiple tumor cell lines in one well or normal cells in a multiplex assay format, so that reactivity could be assessed against multiple cell lines at once. Additionally, increased assay sensitivity may be gained by using fluorescent rather than colorimetric detection of antibody-antigen binding. Moreover, with sufficient correlations with clinical and other diagnostic parameters, this cell-based ELISA may potentially be investigated as a tool to inform on patient outcomes, responses to therapies, and to guide clinical decisions.

Strikingly, it was found that melanoma-reactive antibody cultures were observed in all six patients studied, and this prevalent response merits further exploration. Correlating clinical parameters with degree of antibody response to cancer could guide the selection of patients capable of providing the most abundant source of anti-melanoma antibodies. Additionally, monitoring the memory B cell repertoire of patients can provide insights into the mature B cell response to cancer, supporting the clinical relevance of activating this less-frequently examined arm of adaptive immunity in cancer.

Chapter 4: Monitoring the Humoral Immune Response to Melanoma[6]

[6] Section 4.2 and Figures 4.1-4.9 and Table 4.1 are reproduced in part or in full from Gilbert, Karagiannis et al. 2011.

4.1 Introduction and Aims

The humoral immune response to melanoma has been evidenced by the presence of anti-tumor antibodies in the serum of patients in reports dating back over 40 years (Lewis, Ikonopisov et al. 1969; Sahin, Türeci et al. 1995; Old and Chen 1998; Stockert, Jäger et al. 1998). These serological studies have provided some insight into temporal antibody responses present in the blood at a given time against patient autologous tumor cells and recombinant forms of well-defined melanoma antigens. Further interrogation of the anti-tumor antibody compartment of patients from memory B cells could complement such serological studies and potentially provide additional perspective into the presence and frequency of circulating memory B cells in patient peripheral blood. In principle, these memory B cells may be activated to mount an anti-tumor antibody response. To achieve this, the cell-based ELISA (developed in Chapter 3, Section 3.3.3) was utilized to assess the reactivity of antibodies from circulating memory B cells derived from both patients and healthy volunteers to melanoma cells. Evaluation of memory B cell antibody responses to non-autologous melanoma cells utilizing the cell-based ELISA may confer some advantages in evaluating humoral responses to melanoma over the examination of such responses against autologous melanoma cells or a few pre-defined recombinant melanoma antigens. By measuring antibody reactivity to whole non-autologous cells, overall reactivity to antigens present on melanoma cells can be assessed rather than just a few melanoma antigens or rare antibodies which only recognize autologous cells. Monitoring the circulating human memory B cell compartment of patients for anti-tumor IgG antibodies can permit the characterization of the circulating B cell repertoire of individuals and

would allow for further insight into the breadth of mature B cell responses to malignancy.

This chapter will also examine a novel aspect of humoral immune responses in the cutaneous tumor microenvironment. B cells have been found to infiltrate both primary and metastatic melanoma tumors and it has been suggested that this infiltration may hold some significance in melanoma prognosis (Clemente, Mihm et al. 1996; Ladányi, Kiss et al. 2011; Erdag, Schaefer et al. 2012). These studies have primarily identified B cells by pan-B cell markers such as CD19+ or CD20+ cells and, most likely, this B cell infiltration is comprised of multiple B cell subsets, and each subset may hold distinct roles in the tumor microenvironment (Staquicini, Tandle et al. 2008; Schreiber, Old et al. 2011). Tumor-specific antibodies have been detected from B cells in melanoma tumors (Kirkwood and Robinson 1990; Yeilding, Gerstner et al. 1992). However, the direct impact of tumor-specific antibodies in providing a significant anti-tumor immune response in the tumor is not clear. The effector mechanisms of such tumor-specific antibodies may be weakened in the tumor microenvironment by mechanisms of immune escape such as expression of inhibitory FcγRs (FcγRIIB) on melanoma cells which may function as a decoy rendering the Fc region of anti-tumor antibodies inert (Cassard, Cohen-Solal et al. 2008; Schreiber, Old et al. 2011). Also, it is possible that other regulatory factors, such as cytokines, in the tumor microenvironment may contribute to the alteration of the anti-tumor antibody response.

Individuals with metastatic melanoma and some other cancers have been hypothesized to exist in a state of enhanced Th2 driven inflammation (Sheu, Lin et al. 2001; Agarwal, Verma et al. 2006; Nevala, Vachon et al. 2009). In melanoma, the increase of circulating Th2 cytokines such as IL-4, IL-5, -10 and IL-13 has been

associated with tumor burden (Nevala, Vachon et al. 2009) . The inflammatory environment of tumors, such as the presence of Th2 cytokines is thought to be pro-tumor by the upregulation of VEGF by tumor cells, which contributes to tumor angiogenesis (Nevala, Vachon et al. 2009). These Th2 cytokines are also associated with IgE class switching (IL-4) and the production of IgG4 antibodies (IL-10, IL-4) (Jeannin, Lecoanet et al. 1998); here it is hypothesized that Th2 cytokines present in the tumor microenvironment, such as IL-4 and IL-10, may modulate anti-tumor antibody responses in tumors resulting in the increased production of IgG4 secretion from B cells. In order to test this hypothesis and examine possible IgG subclass polarization in the tumor, the production and proportional distribution of the IgG subclasses were measured from B cells residing in metastatic melanoma tumors.

Studying the distribution of IgG subclasses in the tumor microenvironment may provide valuable insight into the local antibody response to melanoma from memory B cells found *in situ*. IgG is comprised of four antibody subclasses, each having different molecular structures and effector functions, with IgG1 being generally thought to have the most potent immune effector function (Bruggemann, Williams et al. 1987). Changes in the proportions of IgG subclass levels in the tumor, such as decreased levels of IgG1 and increased levels of IgG4, may result in the dominance of such a subclass reported to be a poor activator of complement (van der Zee, van Swieten et al. 1986), to have low affinity to relevant FcγRs involved in effector function (Bruhns, Iannascoli et al. 2009), and to possibly have low affinity to antigens due to Fab arm exchange resulting in the inability to crosslink antigens (van der Neut Kolfschoten, Schuurman et al. 2007). Thus, it is hypothesized that a modulation of IgG subclass in the tumor by Th2 cytokines

towards IgG4 production may weaken the tumoricidal properties of memory B cells.

The main objective of this chapter is to characterize the antibody response to melanoma from memory B cells in both the periphery and tumor microenvironment. The specific aims of this chapter are two-fold:

1. To evaluate the prevalence of the melanoma-reactive B cell compartment in circulating B cells from 21 patient cohorts comprised of individuals diagnosed with Stage I, II, III and IV melanoma.
2. To compare the IgG subclass composition from memory B cells in the peripheral blood and the tumor microenvironment in order to investigate possible local modulation of the humoral immune responses.

4.2 Monitoring Peripheral Blood B cells for Melanoma-reactive IgG Antibodies

Prior to evaluating the reactivity of antibodies secreted by B cells among a patient cohort, the presence and frequency of the memory B cell compartment was first evaluated. CD27+ was used as a marker of memory B cells (Klein, Rajewsky et al. 1998) and the circulating memory B cell populations were compared among patient groups and healthy volunteers. Following these characterizations, evaluations of memory B cell responses were performed in patients such as those evaluating antibody responses to melanoma in relation to disease progression and

those estimating of the frequency of memory B cells producing melanoma-reactive antibodies.

4.2.1 Analysis of Circulating CD27+ Memory B cell Compartments

Disruption in memory B cell homeostasis has been documented in autoimmune diseases such as Systemic Lupus Erythematosus (SLE) and HIV and more recently in cancer (Odendahl, Jacobi et al. 2000; De Milito, Mörch et al. 2001; Carpenter, Mick et al. 2009). Carpenter and colleagues observed that patients with advanced melanoma and with other advanced breast, brain, and pancreatic tumors had a reduction in the proportion of peripheral blood CD27+ cells compared to healthy volunteers, and that these CD27+ memory cells showed a reduced response to B cell activation signals and ability to stimulate T cells in *ex vivo* stimulation assays (Carpenter, Mick et al. 2009). These results suggest a weakened humoral immune response in patients with Stage IV melanoma.

To evaluate if there was a reduction of circulating memory cells as a function of disease progression, the CD27+ memory B cell compartment was compared among healthy volunteers, non-metastatic (Stage I & II) and metastatic (Stage III & IV) melanoma patients by flow cytometric analyses (see Chapter 2, Section 2.6.3.1). A significant ($P<0.05$) decrease in the percentage of CD27+ B cells from the mature (CD22+) B cell pool (mean %CD27+ cells in CD22+cell population ± SEM) was observed for both non-metastatic (16.5 ± 1.8) and metastatic melanoma (16.2 ±1.5) patients compared to healthy volunteers (45.8 ± 9.4) (Figure 4.1). The results described here for the healthy volunteer and metastatic melanoma patients' memory B cell populations were similar to the more exhaustive study

performed by Carpenter and colleagues (Carpenter, Mick et al. 2009). These results also provided additional information on alterations of the B cell phenotype in early points of malignancy by the examination of the memory B cell compartment of individuals with non-metastatic disease.

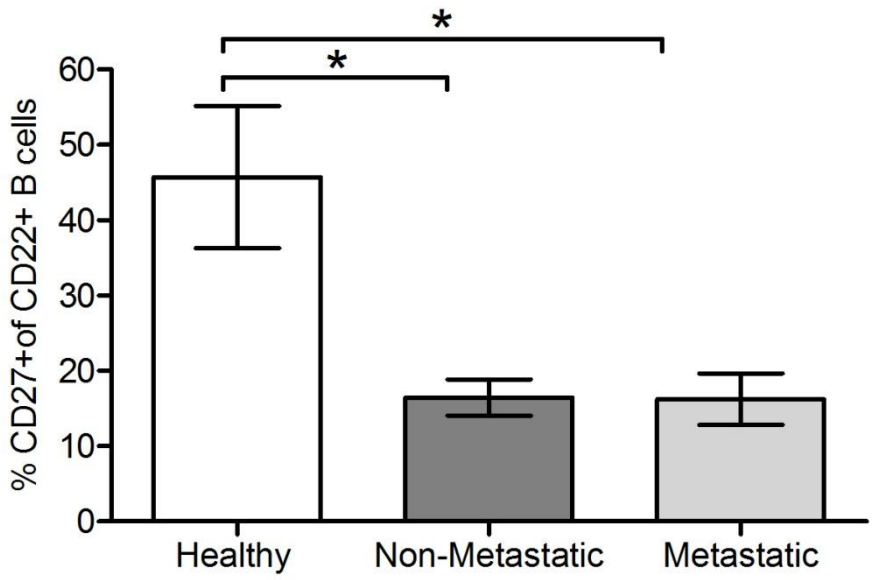


Figure 4.1 Estimations of the peripheral blood CD27+ memory B cell compartment in patients diagnosed with non-metastatic and metastatic melanoma compared to healthy volunteers. The percentage of memory (CD27+) B cells in total mature (CD22+) B cell populations was compared among healthy volunteers (n=5) and melanoma patients diagnosed with non-metastatic (n=4) and metastatic melanoma (n=5). Error bars represent standard error of the mean (SEM). Melanoma patients with metastatic and non-metastatic disease were compared to healthy volunteers using a Mann Whitney U Test. *=P<0.05.

Interestingly, no significant difference in the percentage of CD27+ memory B cell populations was detected between metastatic and non-metastatic melanoma patient groups, despite both groups having significantly lower circulating memory B cell compartments than healthy volunteers (Figure 4.1). These results point to a reduction in the memory B compartment in the periphery at earlier stages of

malignancy. This loss of memory B cells in the peripheral blood of cancer patients is not well understood. One possibility would be the sequestering of memory B cells from circulation, which may include the infiltration of memory B cells into tumors. Interestingly, Carpenter and colleagues observed a significant difference among the proportion of CD27+ populations among healthy volunteers, patients with active Stage IV melanoma and those with Stage IV melanoma who had no evidence of disease. In this study, patients with active disease were found to have the most reduced CD27+ populations, followed by those with no evidence of disease and then healthy volunteers (Carpenter, Mick et al. 2009). These findings do not largely support the hypothesis that B cells are primarily being sequestered in the tumors. This is because even though patients with higher tumor burden had the smallest CD27+ circulating B cell compartment, a reduction in memory B cell populations was also seen in those diagnosed with Stage IV melanoma and currently having no evidence of disease and therefore with a tumor burden similar to healthy volunteers. Alternatively, the decrease in memory B cell populations in patients may represent an overall reduction or dysregulation of memory B cells in individuals with melanoma. Here, the loss of circulating memory B cell phenotype has been observed in both patient groups and does not appear to solely be a function of increasing tumor burden.

More importantly in terms of understanding the effects of a reduced memory B cell compartment in melanoma patients on the humoral immune response, the question whether there is a proportionate loss of B cells with anti-tumor reactivity in circulation remains unclear and warrants further evaluation (See Section 4.2.3 for such evaluations). Prior to addressing this question, the characterization of the reactivity of antibodies from circulating memory B cells from both patients and

healthy volunteer will first be examined in order to assess the presence and prevalence of the humoral immune response to melanoma in peripheral blood B cells.

4.2.2 Prevalence of Melanoma-reactive Antibodies from Patient and Healthy Volunteers

The presence and prevalence of circulating B cells containing melanoma-reactive IgG antibodies were evaluated for both patients and healthy volunteers from peripheral blood B cell *ex vivo* cultures. This was achieved by measuring antibody reactivity to melanoma cells using a novel cell-based ELISA (Chapter 3). Antibody reactivity was calculated relative to a non-specific human IgG control in order to compare the anti-melanoma antibody response to metastatic and primary melanoma cells between patients and healthy volunteers, and among patient groups. Absorbance values were normalized using the following formula:

$$Fold\ increase = \frac{Absorbance\ of\ B\ cell\ culture\ supernatant}{Absorbance\ of\ non-specific\ human\ IgG\ control}$$

Fold increase represents antibody responses to melanoma from memory B cells to an array of antigens expressed on melanoma cells.

The prevalence of melanoma-reactive antibodies in patients was compared to healthy volunteers employing the cell-based ELISA. First, the reactivity of antibodies secreted in B cell cultures to metastatic melanoma cells was evaluated for 600 B cell cultures derived from 10 healthy volunteers (60 cultures per individual, individual age ranged from 21-46) and for 600 B cell cultures derived

from 10 patients (n=4 stage II, n=4 stage III, and n=2 stage IV, individual age ranged from 49-88). A significant increase (P<0.001) in the mean reactivity (fold increase) of antibodies to metastatic melanoma cells was found in patient culture supernatants (2.5 fold increase, 95% confidence interval [CI] 2.4 to 2.6) compared to healthy volunteer culture supernatants (1.1 fold increase, 95% CI 1.1. to 1.2) (Figure 4.2, A). Only a few cultures derived from healthy volunteer B cells, approximately 1% of cultures, were measured to have reactivity greater than a 3-fold increase above the IgG control (Figure 4.2, B). This high reactivity of these few cultures may reflect reactivity to proteins not exclusively expressed on melanoma cells. Alternatively, these few cultures may represent false positive results from the ELISA. While healthy volunteers and patients were not age matched due to the inaccessibility of older healthy volunteers, humoral immunity is thought to be enhanced in younger patients (Allman and Miller 2005) and the use of younger individuals in the healthy volunteer group could quite possibility provide an even more rigorous comparison to the humoral immunity of older patients with melanoma. In comparison to healthy volunteer cultures, patient cultures such as those derived from patient M111 were seen to have many cultures with high antibody reactivity to metastatic melanoma cells (Figure 4.2, C). One of these cultures from patient M111 was selected for further expansion and subcloning to produce a monoclonal culture, and subsequently the antibody specificity and functions of one of these patient antibodies was further characterized as detailed in Chapter 5, Section 5.2.2. Overall, a significantly higher reactivity to metastatic melanoma cells was observed from patient cultures compared to those derived from healthy volunteers.

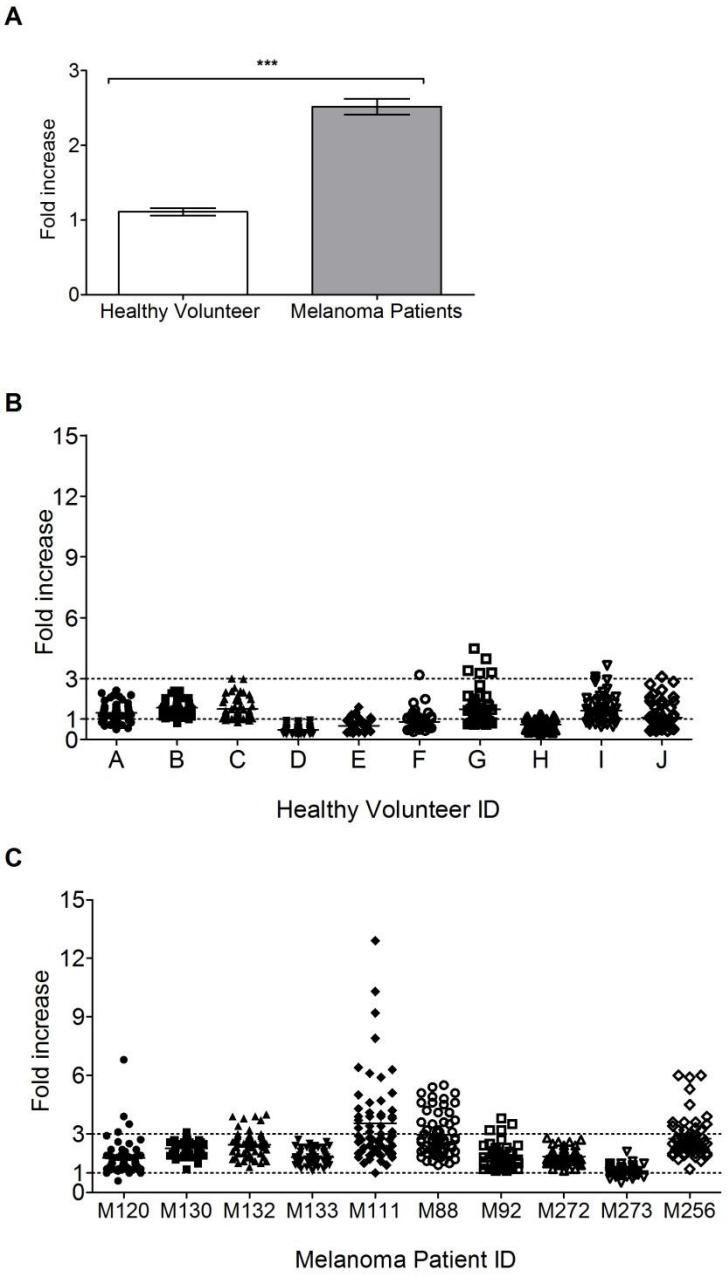


Figure 4.2 Reactivity of antibodies derived from patient and healthy volunteer B cells to metastatic melanoma cells. (A) Mean reactivity of B culture supernatants (n=600) from 10 melanoma patients was compared to the mean reactivity of B cell culture supernatants from 10 healthy volunteers (n=600). Antibody reactivity for each B cell culture was evaluated from individual healthy volunteers (B) and patients (C) and was determined using the cell-based ELISA. Fold increase values represent the absorbance value of each B cell culture relative to the mean absorbance value of a negative control antibody. Error bars in (A) represent 95% CI. A two-sided Student's *t* test was used to compare healthy volunteers and patients (A). *** = $P<0.001$.

Next, the reactivity of antibodies from patient and healthy volunteer B cell cultures was evaluated against WM-115 human primary melanoma cells. A significant (P<0.001) increase was observed in the mean antibody reactivity of cultures (n=600) derived from 10 patients (2.3 fold increase, 95% CI 2.2 to 2.4) to primary melanoma cells compared to cultures (n=600) derived from 10 healthy volunteers (1.0 fold increase, 95% CI 1.0 to 1.1) (Figure 4.3A). Similar to the results obtained for the reactivity of cultures to metastatic melanoma cells, a few cultures (<2%) from healthy volunteers had reactivity to primary melanoma cells greater than 3-fold increase above the non-specific human IgG control (Figure 4.3B). Interestingly, patient B cell cultures overall displayed higher reactivity to metastatic melanoma cells (A-375) than those melanoma cells derived from a primary lesion (WM-115). The patient with the highest number of cultures containing antibodies reactive to primary melanoma cells was patient M273 (Figure 4.3C) who was also seen to have little reactivity to metastatic melanoma cells (Figure 4.2C). The differential binding of antibody cultures to primary and metastatic melanoma cells may reflect the difference of antigen expression on cells during disease progression (Barrow, Browning et al. 2006) although it could equally be due to individual patient responses to specific antigens expressed by different tumor cells. In support of the first possibility, the expression of differentiation antigens has been found to decrease with advancing disease, and antibodies against such antigens may not bind to metastatic cells that lack the expression of these antigens (Trefzer, Hofmann et al. 2006). Overall, in addition to the metastatic melanoma cell line, melanoma patients but not healthy volunteers were also observed to have reactivity against a primary melanoma cell line.

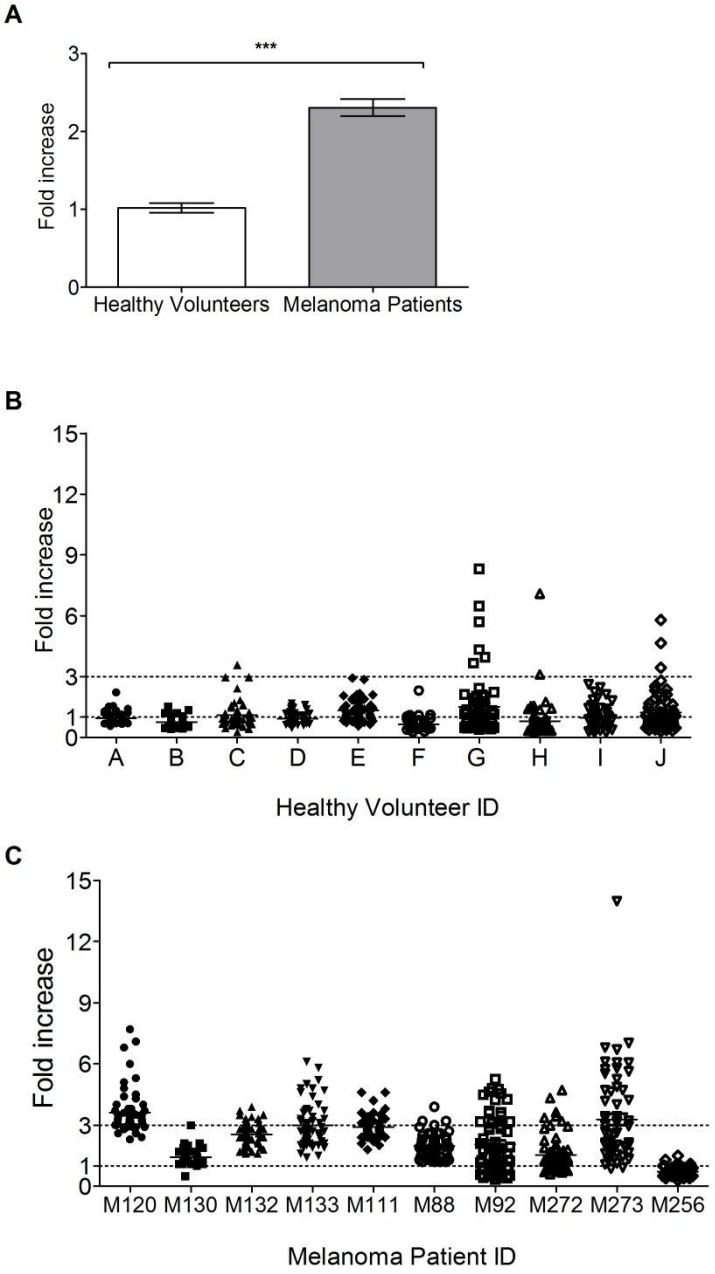


Figure 4.3 Reactivity of antibodies derived from patient and healthy volunteer B cells to primary melanoma cells. (A) Mean reactivity of B culture supernatants (n=600) from 10 melanoma patients was compared to the mean reactivity of B cell culture supernatants from 10 healthy volunteers (n=600). Antibody reactivity for each B cell culture was evaluated from individual healthy volunteers (B) and patients (C) and was determined by the cell-based ELISA. Fold increase values represent the absorbance value of each B cell culture relative to the mean absorbance value of a negative control antibody. Error bars in (A) represent 95% CI. A two-sided Student's *t* test was used to compare healthy volunteers and patients (A). *** = $P<0.001$.

Examining the reactivity of antibodies secreted from patient B cells against primary and metastatic melanoma cells (Figure 4.2 & Figure 4.3) demonstrated the presence of a circulating B cell compartment in this patient cohort containing antibodies reactive to melanoma cells. While a reduction of the CD27+ memory B cell phenotype has been observed in individuals with melanoma (Figure 4.1), B cells activated *ex vivo* from patients were found to secrete antibodies in similar ranges compared to B cells from healthy volunteers (Figure 3.6). The presence of a circulating mature B cell compartment in patients that recognizes melanoma antigens in this patient cohort supports the presence of humoral immune responses against melanoma cells which can be detected following the *ex vivo* activation of patient B cells. The antibody repertoire from this compartment merits further analyses in order to elucidate possible clinical correlations with antibody responses and important prognostic factors. These findings, especially when conducted using larger patient cohorts, will provide valuable insights into the homeostasis of memory B cells capable of mounting an anti-tumor antibody response.

4.2.3 Antibody Response from Memory B cells in Relation to Disease Progression

In order to examine if antibody response measured from *ex vivo* activated memory B cells changed in relation to disease progression, a 21 patient cohort was studied (Table 4.1). The reactivity of antibody cultures (n=1,800) from these patients to A-375 melanoma cells was evaluated using the cell-based ELISA. Patients diagnosed with non-metastatic melanoma were observed to have significantly higher antibody reactivity (measured as fold increase above the negative control antibody) compared to those with metastatic disease (Figure 4.4). While reduced, the presence of melanoma-reactive cultures from patients with metastatic disease was still significantly (P<0.001) higher than that of healthy volunteers (Figure 4.4). These results demonstrate the presence of an anti-tumor B cell compartment among circulating patient B cells that is reduced in metastatic disease. Since antigen composition may change with evolving malignancies (Trefzer, Hofmann et al. 2006), measuring overall antibody reactivity to whole cells rather than just one particular antigen provides a broader perspective of the host humoral immune response to melanoma.

Table 4.1 Reactivity of Patient-derived Antibody-secreting B cell Cultures to Metastatic Melanoma Cells

Stage	Patient ID*	Age	Sex	Ethnicity	Mean fold increase†	95% CI of mean	Maximum fold increase†	% Mean reactive cultures††
I	M119	51	F	Caucasian	1.1	1.0 to 1.2	2.8	2
II	M133	75	M	Caucasian	2.7	2.6 to 2.8	4.2	38
II	M88	70	M	Caucasian	6.1	5.4 to 6.9	17	63
II	M144	69	M	Caucasian	1.6	1.4 to 1.8	4.8	3
II	M256	49	F	Caucasian	2.6	2.5 to 2.8	6.2	22
II	M120	66	F	Caucasian	1.5	1.4 to 1.7	6.8	5
II	M274	63	F	Caucasian	1.6	1.5 to 1.5	3.5	82
II	M271	38	M	Caucasian	1.3	1.1 to 2.2	7.7	2
II	M285	81	M	Caucasian	2.0	1.9 to 1.5	3.7	50
				Mean	2.4	2.2 to 2.7	6.7	33
III	M111	67	M	Caucasian	3.2	3.0 to 3.3	5.8	14
III	M80	54	M	Caucasian	1.8	1.6 to 1.9	3.6	13
III	M92	77	M	Caucasian	1.8	1.6 to 1.9	3.8	97
III	M272	88	M	Caucasian	1.8	1.7 to 1.9	2.8	40
III	M273	68	M	Caucasian	1.3	1.2 to 1.4	2.8	6
III	M164	23	M	Asian	1.0	0.8 to 1.1	2.9	8
				Mean	1.8	1.7 to 1.9	3.6	30
IV	M72	77	F	Caucasian	1.7	1.5 to 1.9	3.3	92
IV	M74	72	F	Caucasian	1.0	0.8 to 1.1	3.5	10
IV	M130	66	M	Caucasian	1.8	1.7 to 1.9	3.1	19
IV	M139	55	F	Caucasian	1.5	1.4 to 1.6	2.6	2
IV	M132	51	M	Caucasian	1.8	1.7 to 1.8	2.7	8
IV	M187	31	F	Caucasian	0.7	0.6 to 0.8	1.3	12
				Mean	1.4	1.3 to 1.5	2.8	24

*Patient ID corresponds to patient number in all figures and text.

†Fold increase values were calculated by dividing the absorbance value of B cell culture supernatants by the absorbance value of a non-specific IgG negative control using a cell-based ELISA.

†† Percent of cultures with absorbance values greater than 75% of a positive control antibody using a cell-based ELISA.

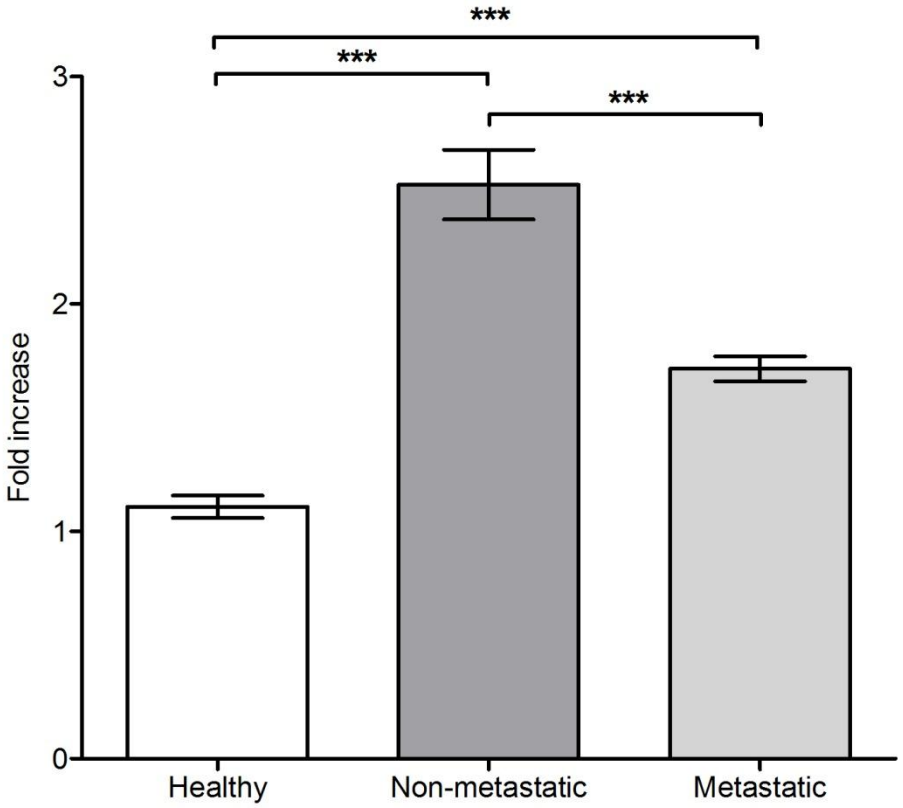


Figure 4.4 The reactivity of IgG antibodies to melanoma cells from metastatic and non-metastatic melanoma patient and healthy volunteer B cell cultures. Antibodies derived from peripheral blood B cell cultures established from healthy volunteers (n=10), non-metastatic (n=9), and metastatic melanoma patients (n=12) were evaluated for reactivity to A-375 melanoma cells. Antibody reactivity was measured as fold increase above a non-specific IgG antibody using a cell-based ELISA. Error bars represent 95% CI. Groups were compared using a one-way ANOVA. *** = P<0.001**.**

When antibody responses were assessed in relation to disease stages, it was found that antibody reactivity to metastatic melanoma cells diminished as a function of disease stage (Figure 4.5). A significant reduction in antibody response was observed in Stage IV patients compared to those diagnosed with Stage II and III melanoma (Figure 4.5). These results support the hypothesis that the anti-tumor humoral immune response is present in patient circulation, but it is hindered or modulated with advancing disease, quite possibly due to mechanisms of immune tolerance induced in these patients by tumor cells. The reduced reactivity of antibodies with disease progression may demonstrate weakened immune responses against a set group of antigens on the surface of melanoma cells. Weakened humoral responses with disease progression have also been previously observed in melanoma by serologic studies (Lewis, Ikonopisov et al. 1969), and levels of auto-antibodies to specific antigens in the sera of patients with melanoma and other cancers have been shown to be higher than serum auto-antibody titers from healthy volunteers or those with benign neoplasms (Shimbo, Tanemura et al. 2010; Blixt, Bueti et al. 2011).

The degree by which these tumor-reactive IgG antibodies have undergone extensive somatic hypermutation and have high affinity for their corresponding TAAs remains unclear in this study. Following the future identification of antigens for some patient-derived tumor-specific antibodies, this could be addressed through the measurement of antigen-antibody affinity. Or prior to antigen identification, this could also be assessed through the evaluation of mutations in multiple tumor-specific antibodies from a patient compared to germline IgG4, such as the analysis performed for clone M80_1F2 (Appendix B), to evaluate if patient-derived antibodies are clonally related as evidence of somatic hypermutation.

Such evaluations could inform on the degree by which these patients could generate a potent mature humoral immune response against melanoma cells.

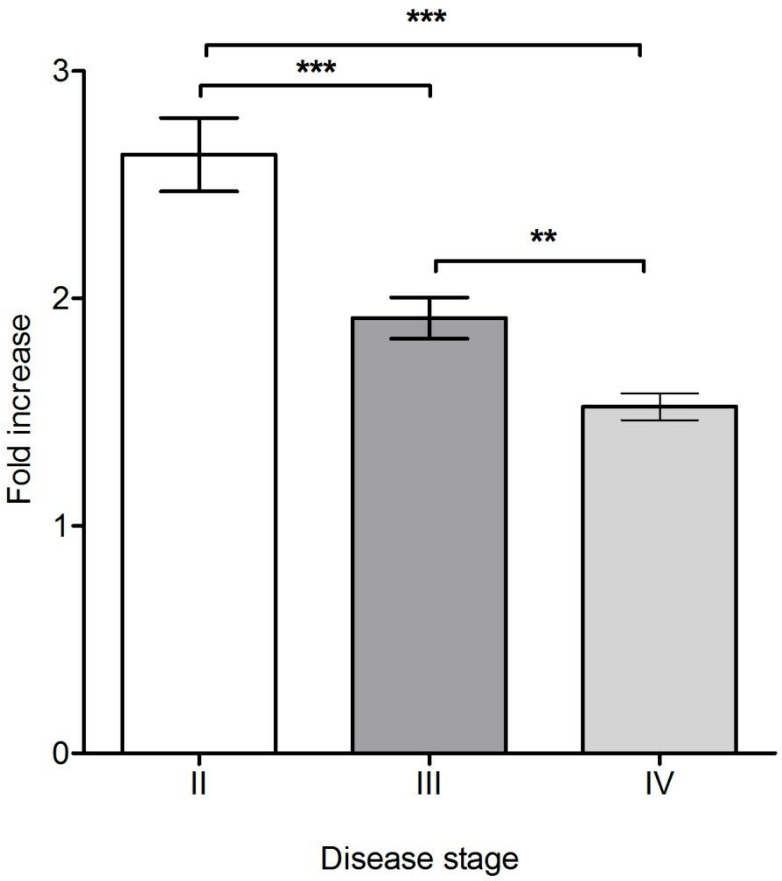


Figure 4.5 The reactivity of patient-derived IgG antibodies to melanoma cells in relation to disease progression. Antibody reactivity to A-375 melanoma cells was measured from patient B cell cultures derived from patients with Stage II (n=8), Stage III (n=6), and Stage IV (n=6) melanoma by the cell-based ELISA. Fold increase values represent absorbance values relative to a negative control antibody (fold increase). Error bars represent 95% CI. A one-way ANOVA was used to compare groups. ** = $P<0.01$; *** = $P<0.001$.

From this 21 patient cohort, a relationship between antibody response and disease stage was observed. Previous serological studies have shown some evidence that serum-resident antibodies are diminished with advancing disease (Lewis, Ikonopisov et al. 1969); the findings described herein suggest that there is a reduction in the humoral immunity to melanoma with advancing disease. This would imply that there may be either a decreased total frequency in memory B cells producing melanoma-reactive antibodies, or a limited capacity of these B cells to secrete antibodies once activated, or a combination of both of these factors. Since this study examines the circulating memory B cell compartment and not serum-resident antibodies, one can exclude the possibility that the loss of tumor-reactive antibodies from the circulation with advancing disease is due to the direct adsorption of antibodies by tumors. It is possible that the observed reduction of antibody responses in patients with advanced disease could be function of the lack of CD4+ T cell help. The presence of CD4+ T cells with specificity to TAAs has been described in melanoma and other cancers and furthermore has been described to correlate with antibody responses (Disis, Calenoff et al. 1994; Gnjatic, Atanackovic et al. 2003; Vence, Paluka et al. 2007). It could be possible that the lack of sufficent CD4+ T cell responses to tumor antigens could result in the ablation of memory B cells in the presence of some tumor antigens (Savelyeva, King et al. 2005) leading to a reduction of antibody responses such as those observed herein for patients with advancing disease. These results described herein support the premise that the anti-tumor humoral immune response is present in patient circulation but perhaps hindered or modulated with advancing disease, possibly due to mechanisms of immune tolerance.

Peripheral blood was only available for one Stage I patient who had a mean antibody response of 1.1 fold increase (Table 4.1), which was comparable to those results obtained for healthy volunteers (Figure 4.2). Patients in the very early stages of melanoma, such as Stage I, could be speculated to have low antibody responses due to localized disease and limited exposure to tumor antigens. However, here no conclusions on tumor reactivity of patient B cells in very early disease setting could be drawn due to sampling limitations.

Additional parameters such as treatment, age, sex, and rate of disease progressions (in a short term 6 months to 2 year follow up) were explored for this patient cohort. No correlations between antibody response and these parameters were found. Whether or not antibody responses can be used as prognostic factors remains unclear. One patient of interest in this study, M120, was diagnosed with Stage II melanoma and remained in remission for 5 years, clear of active disease. While this patient had similar overall mean reactivity to melanoma cells compared to patients such as M130 and M132 who died in the course of this study, this patient was observed to have the second highest maximum antibody response from one B cell culture (Table 4.1). This high antibody response observed in one B cell culture could possibly represent the presence of a melanoma-specific clone with high reactivity to melanoma cells in this patient. Also of note was a patient (M72) with Stage IV melanoma who is a long term survivor (8 years) with stable disease who was found to have the highest percentage of tumor reactive cultures (92%) among the patient cohort (Table 4.1). While no clear prognostic factors of antibody responses emerged from this patient cohort, retrospective analysis aimed at evaluating prognostic factors may provide further insights into the clinical significance of host humoral immune responses to melanoma.

Autoantibodies to some tumor antigens have been shown to correlate with lower incidence of metastases or relapse, with follow-up of > 10 years in other cancers such as lymphoma and breast carcinoma (Ait-Tahar, Damm-Welk et al. 2010; Blixt, Bueti et al. 2011). Additionally, the evaluation of antibodies to TAAs may lead to the early detection of some cancers such as lung, colon and breast cancers (Ran, Hu et al. 2008; Zhong, Coe et al. 2006; Zhong, Ge et al. 2008). Thus, a longer term monitoring of this patient cohort or an extended study incorporating a larger cohort of patients may provide insights into components of the humoral immune system that could hold clinical relevance in melanoma, such as diagnosis or prognostic factors for patients with high or low antibody responses. Also the degree to which a patient can mount an antibody response may provide insights into the selection of therapy, or in the prediction of responses to therapies. For example, it has been reported that treatment with the anti-CTLA-4 antibody, Ipilimumab, led to the serological enhancement of antibodies against the cancer testis antigen NY-ESO-1 (Yuan, Gnjatic et al. 2008); thus, patients with broad antibody responses/repertoires may make good candidates for immunotherapeutic strategies such as those at enhancing T cell responses which in turn could enhance the humoral immune response against TAAs on cancer cells.

4.2.4 Frequency Estimations of Patient B cells Producing Melanoma-reactive Antibodies

The prevalence of antibody cultures containing melanoma-reactive antibodies was approximated for a 21 patient cohort by estimating the percentage of melanoma-reactive cultures. Melanoma cultures were defined as reactive if the absorbance

value of the culture was greater than 75% of the positive control antibody when tested in the cell-based ELISA (criteria detailed in Chapter 3, Section 3.4.2). Using these criteria, it was estimated that 28% of the patient B cell cultures, each originating from 500 cells, contained memory B cells with reactivity to metastatic melanoma cells (Figure 4.6). A similar frequency of melanoma-reactive antibodies was not seen from a cohort of 10 healthy volunteers (Figure 4.6). These results point to the low to moderate numbers of circulating memory B cells in the peripheral blood of patients that are capable of producing anti-melanoma antibodies, although the presence of such tumor-reactive antibodies in patient B cells does not necessarily translate to potent anti-tumor antibody responses in the melanoma cutaneous tumor microenvironment.

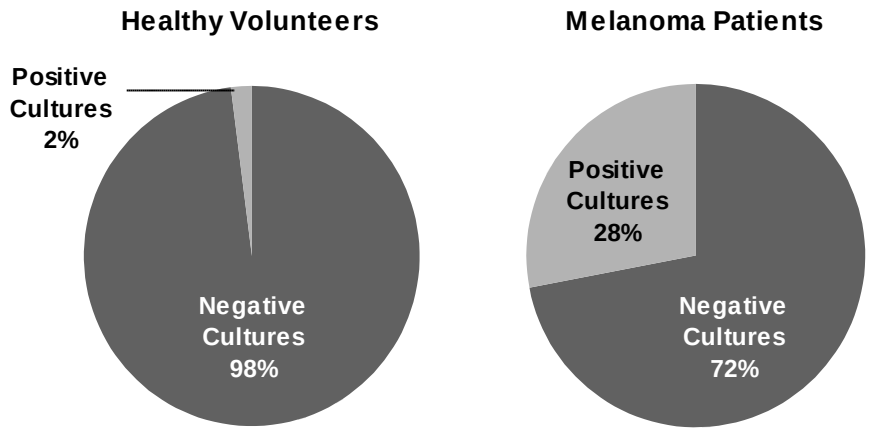


Figure 4.6 Estimation of the frequency of B cell cultures containing antibodies that bind to melanoma cells. The percentage of positive cultures was estimated for healthy volunteers (n=10) and melanoma patients (n=21) using the cell-based ELISA using A-375 melanoma cells. Positive cultures were calculated from the number of cultures originating from 500 B cells which had absorbance values above 75% of the positive control antibody.

Next, the frequency of memory B cells producing antibodies that react with melanoma cells was estimated from a Stage II melanoma patient by performing LDAs using a similar approach to those previously performed by others (Pinna, Corti et al. 2009). The frequency of B cells with melanoma-reactive antibodies was calculated from patients by plating B cells from the same individual at varying densities, culturing cells for 18 days, and then calculating the percentage of culture supernatants with no reactivity to cells/non-reactive cultures (defined as cultures <75% positive control absorbance) using the cell-based ELISA. Following the calculation of the percentage of non-reactive cultures from each density of plated B cells (ranging from 125-10,000 B cells) and performing linear regression analysis on these results, from the Poisson distribution one can estimate that when 37% of the wells have no reactivity to melanoma cells, each well on average had one B cell with melanoma-reactive antibodies. Thus the frequency of melanoma-reactive antibody producing B cells was estimated from the density at which 37% of the wells have no reactivity to melanoma cells under the assumption that a reactive culture represented one B cell clone (see Chapter 2, Section 2.7.5 for further detail of the method). Patients with Stage II melanoma were selected for these analyses since they were predicted to have the highest frequency of melanoma-reactive B cells (Figure 4.5). Individuals with Stage II melanoma would thus require the least total number of B cells and least amount of patient blood; therefore frequency studies were conducted with samples from this cohort. LDAs provided a tool to estimate the frequency of B cells from Stage II patients that were capable of producing antibodies reactive to cells of interest, such as melanoma cells or normal skin cells such as melanocytes.

The frequency of B cells producing antibodies that react to a metastatic melanoma cell line (A-375) were estimated for patient M256 (Figure 4.7). For this patient, the frequency of B cells that produce antibodies that react to A-375 cells was estimated to be 1 in 1790 B cells. Reactivity of antibodies from the same supernatant was also evaluated against melanocytes but the same frequency of reactive antibodies to A-375 cells was not seen (Figure 4.7). In fact, B cells plated at the same densities had almost no reactivity to melanocytes compared to A-375 cells. These results suggest that a much lower frequency of antibodies exist in patient B cells that bind to normal cells of the same origin.

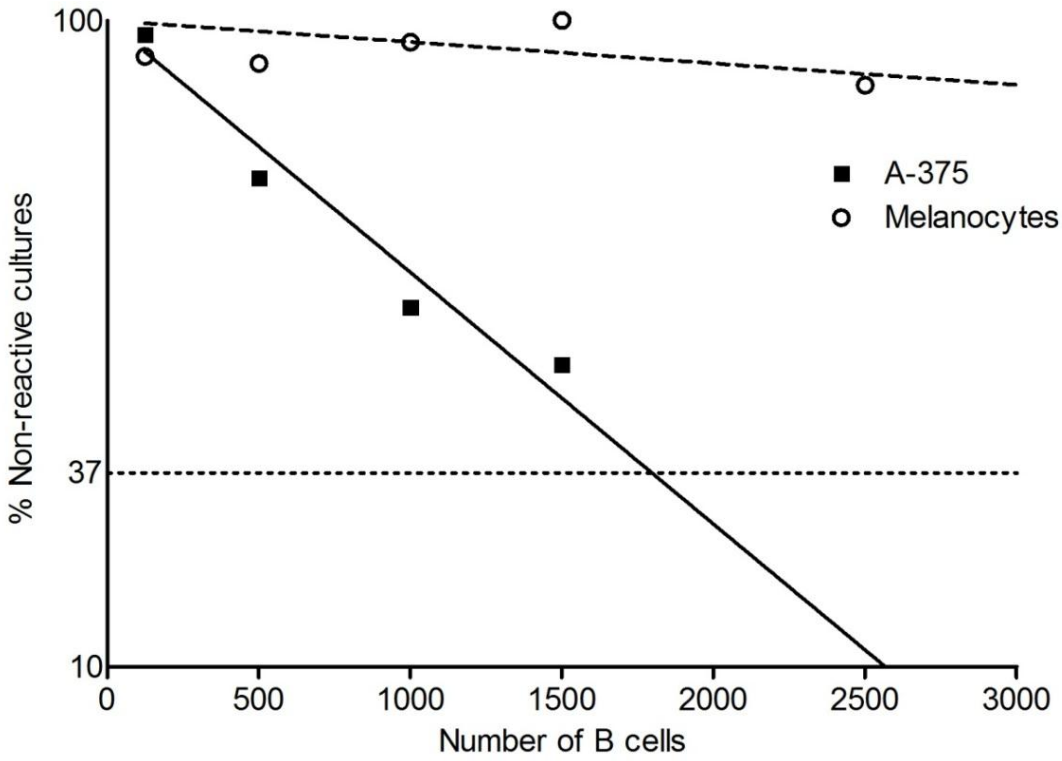


Figure 4.7 Estimations of the frequency of B cells producing antibodies that bind to metastatic melanoma cells compared to melanocytes from a Stage II patient. The frequency of *ex vivo* cultured B cells that produced reactive antibodies to cells of interest was estimated using limiting dilution assays for one patient (M256). The percentage of non-reactive cultures was calculated from the number of B cell culture wells in the cell-based ELISA with absorbance values >75% of the positive control antibody. Reactivity to both the A-375 metastatic melanoma cells and melanocytes was evaluated.

Next, the frequency of melanoma-reactive antibodies from B cells was compared across multiple cell lines originating from metastatic and primary melanoma tumors. Utilizing three metastatic melanoma cell lines (A-2058, A-375 and SK-MEL-28) and one primary melanoma cell line (WM-115), the estimation of the frequency of patient B cells that produce antibodies that react with different tumor cells was made for patient M256. Interestingly, similar frequencies among the metastatic melanoma cell lines (A-2058, 1 out of 1,170 B cells; A-375, 1 out of 1790 B cells; and SK-MEL-28, 1 out of 1,650 B cells) were observed (Figure 4.8). However, frequency of reactive B cells to the WM-115 primary melanoma cell line could not be estimated based on the density of B cells chosen and would likely be less frequent than the rates measured for metastatic melanoma cells for this individual (Figure 4.8). This may be a result of the antibodies being against proteins expressed in metastatic disease and not in earlier stages of malignancy. Alternatively, the results may be related to a lack of reactivity to the particular melanoma cell line and not informative of disease stage.

Lastly, to evaluate if there was a difference in the frequencies of B cells producing melanoma-reactive antibodies between patients, the frequency of B cell cultures capable of producing melanoma-reactive antibodies from 2 patients diagnosed with Stage II melanoma were compared (M256 and M271, Table 4.1). In comparing the B cells from these two patients, similar frequencies of cells producing anti-melanoma antibodies were estimated against the A-375 metastatic melanoma cell line (Figure 4.9) for patient M256 (1 in 1,790 B cells) and patient M271 (1 in 2,430 B cells) by LDA. These frequency estimations corresponded to previous descriptions of antibody responses, where it was found that patient M256 had higher mean antibody reactivity values and a more frequent B cell

mediated antibody response to melanoma than B cell cultures from patient M271, who was also diagnosed with Stage II disease (Table 4.1 & Figure 4.9).

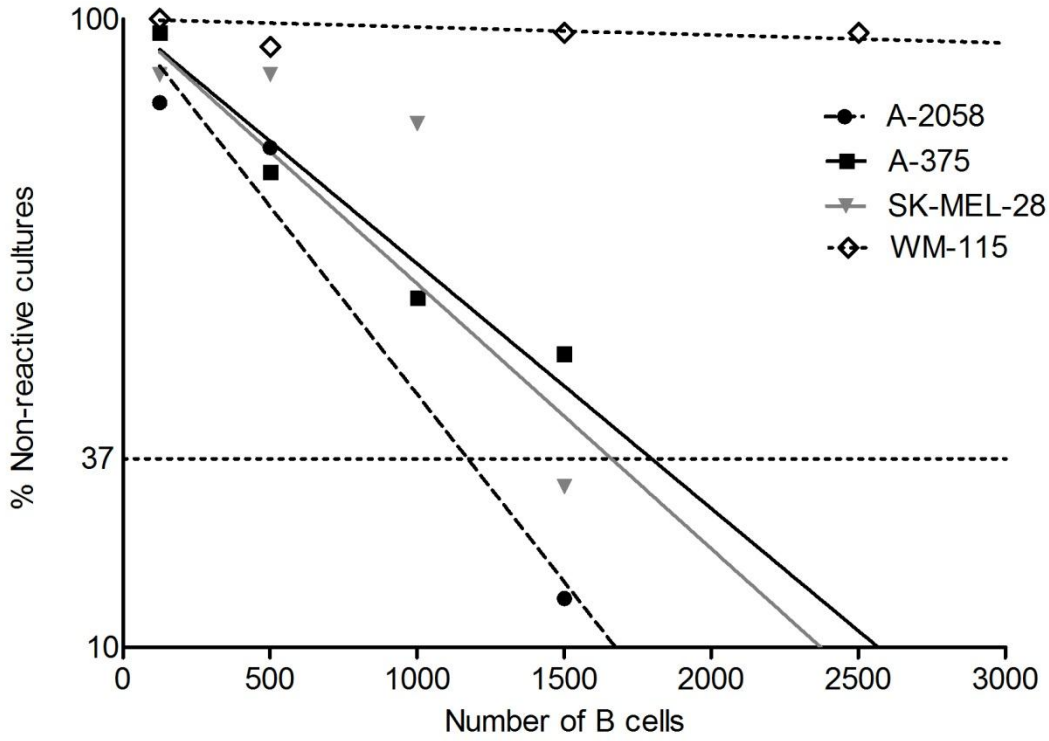


Figure 4.8 Estimations of the frequency of B cells producing melanoma-reactive antibodies to three metastatic and one primary melanoma cell line from a Stage II patient. Using LDAs, the frequency of B cells that produced melanoma-reactive antibodies in culture was estimated for one patient (M256) against three metastatic melanoma cell lines (A-2058, A-375, and SK-MEL-28) and one primary melanoma cell line (WM-115). The percentage of non-reactive cultures was calculated from the number of B cell culture wells in the cell-based ELISA with absorbance values >75% of the positive control antibody.

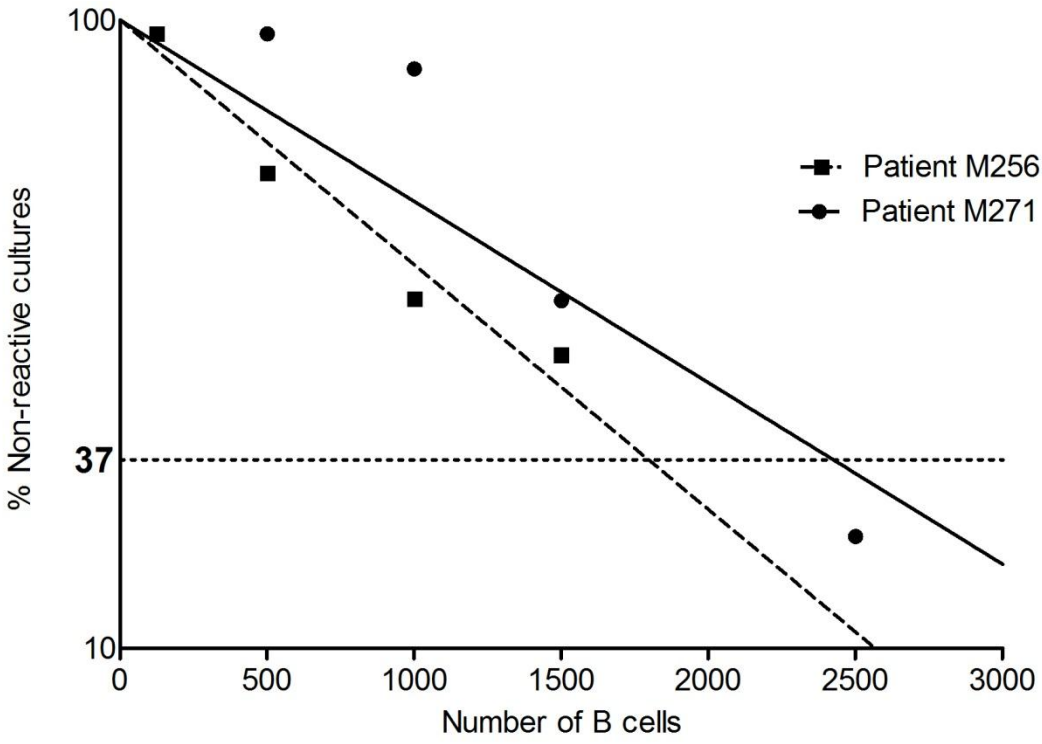


Figure 4.9 Frequency of B cells producing melanoma-reactive antibodies from two patients with Stage II melanoma. Using LDAs, the frequency of B cells that produced melanoma-reactive antibodies in culture was estimated for two patients (M256 and M271) against A-375 metastatic melanoma cells. The percentage of non-reactive cultures was calculated from the number of B cell culture wells in the cell-based ELISA with absorbance values >75% of the positive control antibody.

Considering the array of tumor antigens patients are potentially exposed to and the expected variability in humoral immune responses among patients, these results support the presence of a moderately frequent anti-melanoma antibody compartment in patient peripheral blood memory B cells. Frequencies were estimated to be in the range of 1 in 2000 memory B cells. By comparison, these responses were less frequent than rates described for memory B cell-derived antibodies against toxoid, measles virus antigens and Varicella Zoster virus antigens, which have been reported to be 1 in 400, 1 in 50, and 1 in 585, respectively, for individual donors (Pinna, Corti et al. 2009). In light of these estimations, the frequency of memory B cells with melanoma-reactive antibodies is lower than the frequency of reactive antibodies following common vaccinations. However, they do represent the presence of long term memory to melanoma in patients.

These studies are the first to report the frequency of tumor-reactive antibody secreting memory B cells in patients with cancer. Knowledge of the frequencies of such cells could be important when considering potential therapeutic approaches to activate memory B cells. Since a correlation between serum antibody titers and the frequency of memory B cells may not necessarily exist (Leyendeckers, Odendahl et al. 1999), the measurement of the frequency of memory B cells capable of producing anti-tumor antibodies could guide the selection of patients and potency of methods required to active these memory B cells or serve as a parameter which would allow the monitoring of patient humoral immune responses over time or patient responses to treatments. The identification and selection of patient groups with high frequencies of memory B cells with natural antibody responses against melanoma antigens may also allow for the selection of

appropriate candidates for immunotherapies aimed at enhancing humoral immune responses to cancer such as vaccines. It is possible that in the future, the memory humoral immune response could be evaluated as a biomarker in cancer.

Thus far, the anti-melanoma IgG antibody response has been characterized from peripheral blood memory B cells. While a humoral immune response to melanoma was observed throughout this 21 patient cohort, some degree of dysregulation of the humoral immune response to melanoma has also been observed in patients, including a reduced CD27+ B cell compartment and less frequent antibody responses with increasing tumor burden. Another potential aspect of dysregulated humoral immunity will be examined next, namely the proportion of IgG subclasses expressed by B cells in circulation and in tumor lesions, which may potentially influence the effectiveness of humoral immunity against tumor cells.

4.3 Relative Proportion of the IgG Subclasses Secreted by B cells

To further explore the humoral immune response to melanoma, the relative proportional production of IgG subclasses from memory B cells was investigated in patients and compared to healthy volunteers. Four IgG subclasses have been named relative to their abundance in sera: IgG1>IgG2>IgG3>IgG4, with each subclass having different affinity to FcγRs on immune cells and distinct effector functions (Bruggemann, Williams et al. 1987; Bruhns, Iannascoli et al. 2009). This section will evaluate the relative IgG subclass proportions from B cells isolated from patient peripheral blood and tumors in order to study subclass composition

in individuals with melanoma. It is hypothesized here that Th2 cytokines, such as IL-4 and IL-10, present in the tumor microenvironment may polarize immunoglobulin production favoring the production of IgG subclasses in tumors with less potent effector functions, such as antibodies of the IgG4 subclass. While not previously described in melanoma tumors, significant levels of IgG4 positive plasma cell infiltration have been very recently described in another cancer, extrahepatic cholangiocarcinoma (biliary tract cancer), and this increase in IgG4+ cells has been proposed to be mediated by the tumor cell production of IL-10 (Harada, Shimoda et al. 2012).

4.3.1 Proportional Production of IgG Subclasses from Peripheral Blood B cells Isolated from Healthy Volunteers and Patients

Relative proportions of the IgG subclasses were first evaluated in peripheral blood B cells from both patients and healthy volunteers. Since the composition of IgG subclass antibodies secreted by the peripheral memory B cell compartment had not been reported, the IgG1-4 subclass composition was determined for healthy volunteer peripheral blood B cells following B cell isolation and *ex vivo* culture (described in Chapter 2, Section 2.4.2) and the concentration of IgG1-4 subclasses in these cultures was analyzed using an in-house adapted IgG subclass ELISA (Chapter 2, Section 2.7.4). This evaluation was also conducted to investigate any potential effects of EBV transformation and TLR9 activation on the proportional production of antibodies of different subclasses secreted in *ex vivo* cultured B cells. It should be noted that the *ex vivo* stimulation techniques were used to culture B cells in these examinations because they are particularly key to the survival of B

cells isolated from blood and other tissues, including those derived from tumors. The IgG subclass composition in healthy volunteer peripheral blood B cells was found to be similar to those values reported for IgG subclass composition in sera of individual normal donors (Table 4.2). Thus the addition of EBV and the TLR9 agonist CpG ODN, used to transformed and activate human B cells, did not appear to significantly affect the proportional production of IgG subclasses in peripheral blood B cell cultures.

Table 4.2 Proportional Production of IgG Subclasses in Healthy Volunteer Serum and Peripheral Blood B cell Cultures

Source	% IgG1	% IgG2	% IgG3	% IgG4
Serum†	60-70	14-20	4-8	2-6
Peripheral blood B cells††	79 ± 8	14 ± 8	4 ± 1	2 ± 1

† Values reported from Hamilton et al. 1987

†† Values represent mean ± one SD and were derived experimentally from healthy volunteers (n=4) and tested in triplicate for each individual.

Next, the IgG subclass composition of antibodies secreted from the peripheral blood B cells of patients with metastatic melanoma was assessed to evaluate whether there was any modulation in IgG subclass proportional expression in circulating B cells within the context of cancer. The IgG subclass composition (mean ± SD) in *ex vivo* metastatic melanoma patient (n=3) peripheral blood B cell culture supernatants for IgG1, IgG2, IgG3, and IgG4 was: 69.7.% ± 16.4%, 22.2% ± 9.3%, 4.2% ±3.8% ,and 4.2% ± 3.3% , respectively (Figure 4.10). No significant statistical difference was found between the IgG subclasses produced by peripheral blood B cells from these patients and healthy volunteers when

employing a two-sided Student's *t* test (Figure 4.10). The similarity of IgG subclass compositions produced from memory B cells between patients and healthy volunteers suggests a possible lack of IgG subclass modulation in the peripheral blood memory B cell compartment of the patients evaluated, although subtle differences between the two groups may be apparent if larger cohorts of patients were examined.

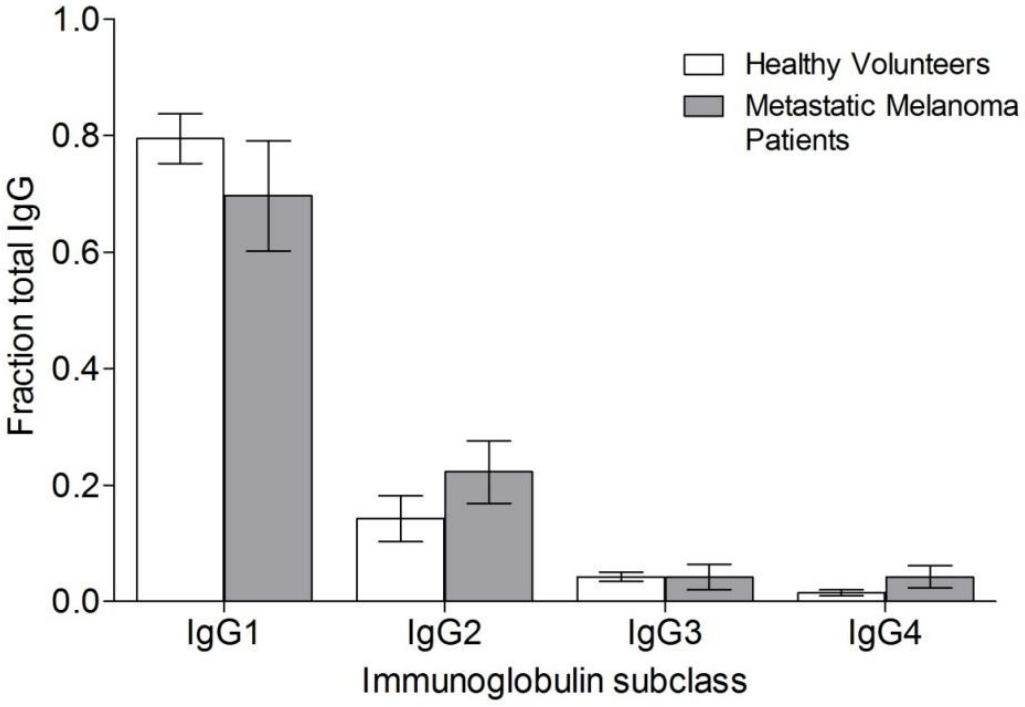


Figure 4.10 Relative proportional production of IgG subclasses from patient and healthy volunteer peripheral blood B cells. B cells isolated from healthy volunteers (n=4) and Stage III metastatic melanoma patients (n=3) were cultured in the presence of irradiated autologous PBMCs, EBV and the TLR9 agonist CpG ODN. Culture supernatants were removed after 10 days and IgG subclass was analyzed by ELISA. Each sample condition was tested in triplicate and values represent mean fraction of total IgG. Error bars represent one SD.

4.3.2 Relative Proportional Production of IgG Subclasses from Tumor-resident B cells

Following the culture of tumor-resident B cells from three melanoma tumor lesions (described in Chapter 2, Section 2.4.3), the composition of IgG subclasses in these cultures was measured by ELISA (See Chapter 2, Section 2.7.4). An increase in the relative proportional production of antibodies of the IgG4 subclass was observed in the two skin tumors (M218 and M221), along with a relative decrease in the proportional production of IgG1 (Figure 4.11) when compared to IgG subclass portions measured from patient peripheral blood (Figure 4.10). These IgG subclass ELISA results from B cells derived from cutaneous tumors from two individuals displayed a striking shift in the proportions of antibody subclasses secreted by tumor-resident B cells. Notably, this shift in antibody subclass composition has not been described before in the melanoma tumor microenvironments and represents a novel finding of this thesis. Interestingly, there has been one report of altered serum IgG4 levels in some melanoma patients, with elevated levels of IgG4 in the sera of a portion individuals diagnosed with later stage disease (Daveau, Pavie-Fischer et al. 1977). No similar reports of altered IgG4 levels in the sera have been reported for other solid tumors or in the microenvironment of the tumor beyond the observation of infiltrating IgG4+ plasma cells in extrahepatic cholangiocarcinoma (Harada, Shimoda et al. 2012). These novel findings of an increase in IgG4-expressing B cells in cutaneous metastases, taken together with the findings of Daveau et al., suggest a potential association between IgG4-producing B cells and melanoma, which may have functional implications.

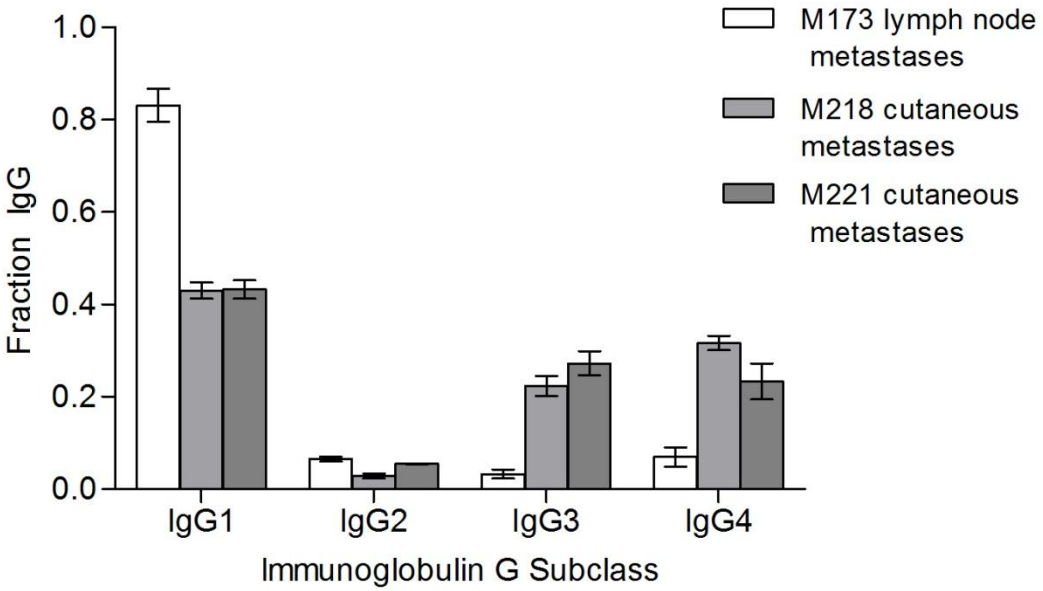


Figure 4.11 Relative proportional production of IgG subclasses from B cells isolated from metastatic melanoma tumors in the skin and lymph nodes. Single cell suspensions were derived from metastatic melanoma tumors found in the skin (n=2) and lymph nodes (n=1) and cultured in the presence of EBV and CpG ODN. Culture supernatants were removed after 18 days and IgG subclass concentrations in culture supernatants were determined by ELISA. Patient B cell cultures were pooled for each patient and then tested in triplicate with values representing the mean fraction of total IgG and error bars representing one SD.

Inversely, the decrease in the production of IgG1 antibodies in the cutaneous metastases from two individuals also suggests a potential mechanism that may modulate the anti-tumor antibody responses in solid tumors. This would be because antibodies of the IgG1 subclass are thought to have the most potent effector functions (Bruggemann, Williams et al. 1987), which would be important for the host to mount an effective immune response against tumor antigen bearing cells. Indeed, IgG is the primary subclass used for cancer immunotherapy (Weiner, Murray et al. 2012). Thus a reduction in IgG1-producing B cells in the tumor could result in a weakened humoral immune response if these antibodies recognized tumor cells. However, the specificity of these IgG1-4 antibodies to melanoma cells remains unknown. Efforts to explore the melanoma cell reactivity of *in situ* tumor resident B cells were attempted, however these were not successful to-date due to irregular availability of tumor samples and also due to technical issues relating to the sensitivity of IgG subclass reagents.

In addition to 2 skin metastases, IgG subclass composition was also evaluated for B cells residing in a non-sentinel lymph node metastasis. The percentage (mean ± SD) of each IgG subclass produced from B cells isolated from a lymph node metastasis was: IgG1=83.2 ± 3.6; IgG2=6.5 ±0.5; IgG3=3.2 ± 1.0; IgG4= 7.0 ± 2.0. Interestingly, the IgG subclass composition was different for B cells derived from a lymph node metastasis compared to those measured from the two cutaneous metastases (Figure 4.11). This may be because only a small fraction of the total B cell population in the lymph metastasis measured for IgG subclass composition was comprised of infiltrating B cells which had already undergone class switching. Unlike the lymph nodes, B cells are not normally found in skin, and the B cells derived from these cutaneous metastases may represent only those B cells

infiltrating the tumor. Additionally, while unexplored, it could be possible that the tumor microenvironment, such as the cytokine levels, of this lesion differed from the skin lesions, resulting in differential proportional productions of IgG subclasses.

Unfortunately, these results represent a very small sample set due to the lack of availability of patient tumors for research purposes, which posed a limitation to this work. To further support these findings, it would be worthwhile to evaluate B cells from more cutaneous and lymph node tumors from other individuals. Notably, while only two cutaneous tumors were available for study during the course of thesis, similar IgG subclass composition between these two patients, M218 (IgG1=43.1 ± 1.7; IgG2=2.8 ± 0.5; IgG3=22.3 ± 2.2; IgG4= 31.6 ± 1.5) and M221 (IgG1=43.3 ± 2.0; IgG2=5.4 ± 0.0; IgG3=27.2 ± 2.6; IgG4= 23.3 ± 3.9), was measured. These findings support the idea that the skewing of relative IgG subclass proportions in the cutaneous metastases is not a one-off finding from rare patient, since it was observed in 2 out of the 2 patients evaluated.

In summary, an increase in the mean relative proportional production of antibodies of the IgG4 subclass was found between B cells isolated from metastatic patient peripheral blood (4.2%, n=4) and from metastatic cutaneous tumors (27.5%, n=2). While matched samples obtained at the same time from individuals were not available for an evaluation between tumors and peripheral blood, levels of IgG4 production was still found to be increased in cutaneous tumors compared to those values reported to be normal in serum and patient blood from those with metastatic disease. The differences in antibody subclasses in blood versus the tumor could possibly be attributed to the increased presence of melanoma cells in

cutaneous tumors compared to peripheral blood, resulting in a different cytokine environment, which will next be explored.

4.3.3 Th2-associated Cytokines and Polarization of IgG Subclasses in the Presence of Melanoma Cells

To elucidate if melanoma cells contributed to the polarization of IgG subclass expression in cutaneous tumors, B cells were co-cultured in the presence or absence of metastatic melanoma cells, and IgG subclass composition from each condition was then evaluated in these *ex vivo* co-cultures (experiments preformed in collaboration with Dr. Panagiotis Karagiannis, KCL). Patient B cells along with irradiated autologous PBMCs were co-cultured in the presence or absence of A-375 melanoma cells at a ratio of 1:5:10 for B cells, PBMCs, and melanoma cells, respectively. After 5 days in culture the proportional production of antibody subclasses was measured by ELISA along with Th2 cytokines IL-4 and IL-10 which were measured using a Luminex multiplex cytokine assay (Millipore). In these co-culture experiments, a proportional decrease in IgG1 secretion was seen along with an increase in IgG4 secretion upon the addition of metastatic melanoma cells together with irradiated PBMCs (Figure 4.12A), which was in concordance with the results obtained for IgG subclass composition in skin metastases. The production of total IgG levels in these co-culture experiments were similar (Figure 4.12). A polarization of IgG subclass production towards IgG4 was not seen in co-cultures without melanoma cells, such as those with just B cells and PBMCs, suggesting that tumor cells may have provided a key factor that influenced subclass expression in these experiments (Figure 4.12A).

Further examination of the Th2 cytokine profiles from these co-culture experiments revealed increased levels of IL-10 and IL-4 in cultures where melanoma cells were included (Figure 4.12C). This finding holds significance because IL-10 is known to be secreted by regulatory cells such as Tregs and alternately activated macrophages in the tumor microenvironments, but it is also secreted by melanoma cells (Dummer, Bastian et al. 1996; Couper, Blount et al. 2008; Itakura, Huang et al. 2011). The secretion of IL-10 by melanoma cells in addition to other immune cells such as T cells may result in the concentration of IL-10 in the tumor microenvironment, which would favor IgG4 production. The IL-10 cytokine has been associated with increased production of IgG4 and decreased IgG1 production in culture (Satoguina, Weyand et al. 2005), and it is thought to augment IL-4 induced IgG4 class switching by B cells (Jeannin, Lecoanet et al. 1998). It is therefore conceivable that elevated levels of the Th2 cytokines, IL-4 and IL-10, may modulate antibody subclass production in B cells residing in cutaneous tumors, potentially favoring an increased production of IgG4 in the tumor microenvironment.

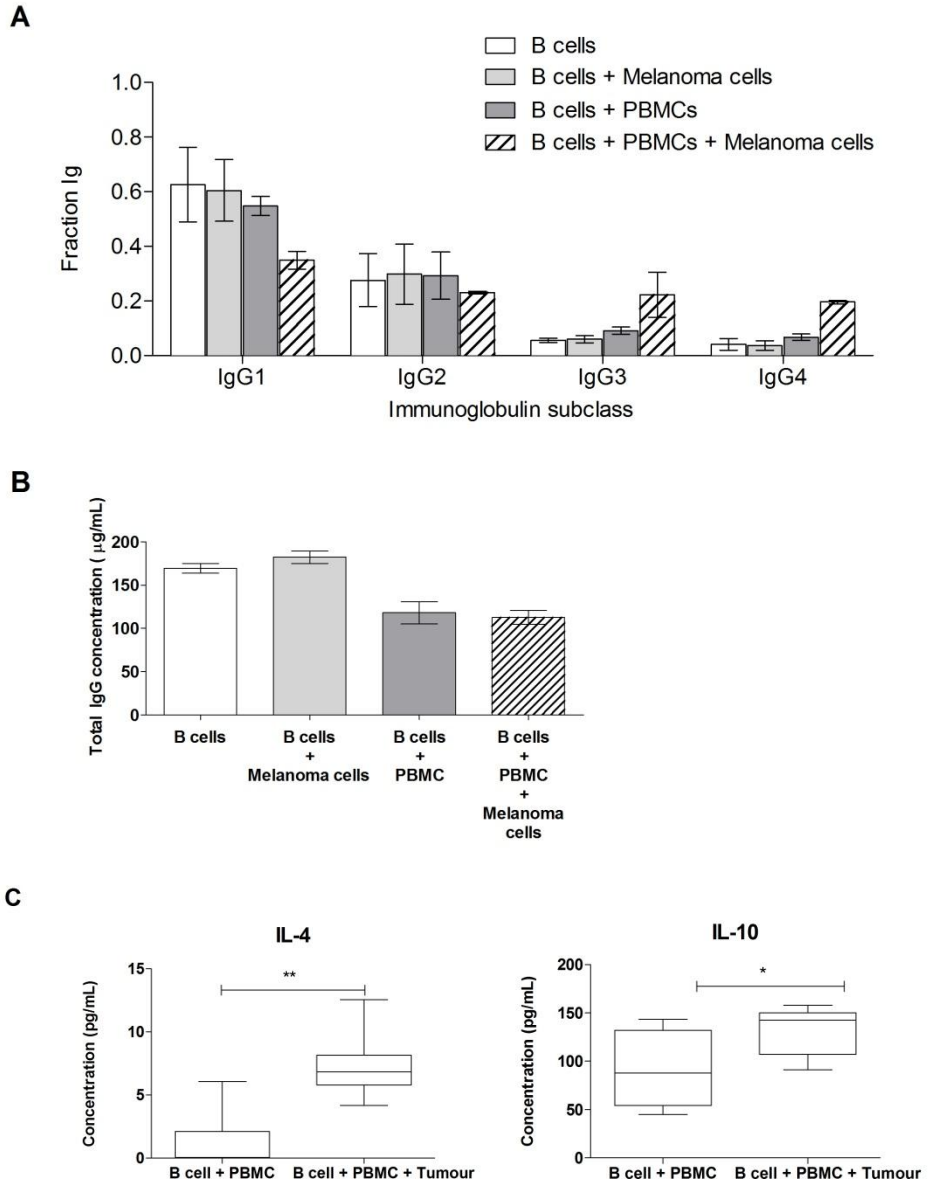


Figure 4.12 Co-culture assays evaluating IgG subclasses and cytokine composition in the presence of melanoma cells. (A) The fraction of each IgG subclass in patient peripheral blood B cell cultures with and without PBMC and melanoma cells was evaluated by ELISA. (B) Comparison of the total amount of IgG secreted in cultures. Values represent mean ± SD of 3 independent experiments preformed in triplicates. (C) Concentrations of selected Th2 cytokines, IL-10 and IL-4, secreted in cultures measured using Luminex multiplex cytokine assays (Millipore). Box plots represent minimum, maximum, and mean values. Results of two assay conditions were analyzed using the Mann Whitney U test (IL-4: * P<0.01; IL-10: ** P<0.01, n=9 replicates per co-culture condition). Experiments were performed in collaboration with Dr. Panagiotis Karagiannis, KCL.

Elevated levels of IgG4 in the tumor microenvironment may present a mechanism by which the tumor and other IL-10 producing immune cells can polarize the humoral response and reduce the potency of antibody functions. First, the polarization towards IgG4 antibody production, can result in elevated levels of an antibody class thought to have the weakest effector functions, manifested by inhibition of complement activation (van der Zee, van Swieten et al. 1986), low affinity to key Fc receptors involved in cell mediated cytotoxicity (Bruhns, Iannascoli et al. 2009) and inability to crosslink identical antigens due to Fab arm exchange properties (van der Neut Kolfschoten, Schuurman et al. 2007). If these IgG4 antibodies are melanoma-reactive, it is possible they may also compete with melanoma-reactive antibodies of the IgG1 class, and once bound to tumor cells they may contribute to a less potent anti-tumor response compared to IgG1. Conversely, these IgG4 antibodies could occupy FcγRs on immune cells present in the tumor preventing the engagement of these cells by other antibodies of more potent antibody subclasses to mediate cytotoxic effects against the tumor.

Taken together, the functional properties of IgG4 could contribute to weakened humoral immune response in the cutaneous tumor microenvironment and may represent a mechanism of tumor escape from the host humoral immune response. These findings have been subsequently explored in mouse models of melanoma which were employed to compare the efficacy of antibodies of the IgG1 and IgG4 classes targeting the HMW-MAA antigen, and the impact of the presence of IgG4 antibodies on otherwise potent IgG1-mediated anti-tumor responses *in vitro* and *in vivo* (Karagiannis & Gilbert, manuscript under revision).

4.4 Conclusions

A prevalent memory B cell antibody response to melanoma was observed in this study, despite the reduction in the CD27+ memory B cell compartment in patients. These observations provide evidence that long-term humoral memory responses to melanoma are present in patients, even in those with non-metastatic disease. These early host humoral immune responses to cancer could quite possibly have significant anti-tumor effects in patients; indeed lymphocyte infiltration into the primary melanoma tumors has been associated with better prognosis (Clark, Elder et al. 1989; Clemente, Mihm et al. 1996). Patients in early stages of malignancy were found to have antibody responses to melanoma from memory B cells at a frequency of approximately 1 in 2,000 B cells. The finding suggests that the memory B cell compartment is mounted in early stages of disease, but the later reduction of anti-melanoma antibody responses detected from this compartment could suggest a weakening of the circulating memory B cell anti-tumor response to melanoma with progressing malignancy. This work complements previous work examining temporal antibody responses in the serum, by contributing to the characterization of antibody responses to melanoma and providing assessments of the reactivity and frequency of long term memory responses to melanoma.

The study of humoral immune responses in the local tumor environments led to the novel finding of elevated levels of IgG4 in the cutaneous metastatic lesions presented in this thesis. This finding may represent a new mechanism by which tumors can evade anti-tumor memory B cell responses. By dissecting antibody subclass composition in the tumor, the possible modulation of humoral immune responses in the cutaneous Th2 microenvironment has been hypothesized. It could be speculated that the presence of elevated levels of IgG4, favored by the

local expression of Th2 cytokines such as IL-4 and IL-10, may result in a weakened anti-tumor humoral immune response by exerting less potent effector functions than the other IgG subclasses or by directly competing with other tumoricidal antibodies in binding to melanoma cells or FcγRs, thereby blocking the effector functions of these antibodies. Such potential immunomodulatory mechanisms of action of IgG4 antibodies are presently being explored by engineering IgG1 and IgG4 antibodies against a melanoma antigen and performing *in vitro* assays evaluating the ability of such antibodies to mediate ADCC and ADCP in different proportions for each subclass, and by evaluating the efficacy of such antibodies in animal models of melanoma using engrafted human effector cells.

In summary, while a long term humoral memory response to melanoma has been described here, the effectiveness and anti-tumor properties of this response may be impaired with disease progression along with changes of IgG subclass composition in the cutaneous tumor microenvironment.

Chapter 5: Novel Antibodies Targeting Melanoma Cells[7]

[7] Section 5.2.2. and Figures 5.2-5.8 are reproduced in part or in full from Gilbert, Karagiannis et al. 2011.

5.1 Introduction and Aims

The discovery of monoclonal antibodies targeting TAAs has generated considerable interest over the last 30 years. Antibodies against TAAs expressed in solid tumors, such Her2/neu and EGFR, have shown considerable clinical and commercial success in the treatment of breast and colorectal cancers (Campoli, Ferris et al. 2010). The mechanisms of action of such antibodies were principally developed to be Fab-mediated resulting in the inhibition of tumor antigen functions. However, the important contributions of Fc-mediated immunological mechanisms of actions of these antibodies in patients are emerging, primarily through the observation of correlations between patient FcγR polymorphisms and clinical responses (Hudis 2007; Strome, Sausville et al. 2007; Musolino, Naldi et al. 2008). The contribution of the Fc portion of therapeutic antibodies towards immune-mediated tumor cell death has also been clearly demonstrated in mouse tumor xenograft models where administration of a fully functional antibody, with a Fab region specific to Her2/neu, was shown to result in a significantly higher reduction in tumor growth compared to a Fc mutated antibody with the same antigen specificity but with impaired FcγR binding (Clynes, Towers et al. 2000). Thus, it has become increasingly clear that the Fc-mediated functions of antibodies can constitute key components of their efficacy (Clynes, Takechi et al. 1998; Clynes, Towers et al. 2000; Nimmerjahn and Ravetch 2008). These findings illustrate the important potential of monoclonal antibodies with specificity to overexpressed antigens on the surface of tumor cells to mediate cellular mechanisms of action against tumor cells such as CDC, ADCC, and ADCP.

Antibodies directed at TAAs, even those that do not directly inhibit antigen function, may additionally provide important vehicles for the specific delivery of other cytotoxic molecules, such as chemotherapeutic agents, radionuclides, and enzymes to tumor cells. Furthermore, such antibodies may serve as important diagnostic tools for disease staging and imaging (Chester, Pedley et al. 2004; Mayer, Francis et al. 2006; Lewis Phillips 2008; Scott, Wolchok et al. 2012). Thus, the discovery of monoclonal antibodies against over expressed TAAs has important and widespread clinical utility.

The results presented in Chapter 4 highlight the presence of an anti-melanoma antibody compartment in patient memory B cells, supporting the claim that tumor cells are recognized by the humoral immune system in individuals with melanoma. While the ability of such cells to mount a significant anti-tumor antibody response in the host is unclear, the striking presence of melanoma-reactive mature B cells in patients and the antibodies these B cells produce may represent a source of reagents whose clinical utility, including evaluations of their therapeutic potential, merits further attention. If identified, characterized, expressed in recombinant form, and administered in sufficient quantities, these patient-derived antibodies may be assessed for their cytotoxic activity against cancer cells as passive immunotherapies. This could be achieved by examining possible mechanisms of action of these antibodies to inhibit antigen function along with evaluations of their potency in activating immune effector cells to mount an anti-tumor response. Therefore, functional assessments of the cytotoxic capacity against tumors of such patient-derived melanoma-specific antibodies warrant further investigation to evaluate their therapeutic potential.

Marketed antibodies for cancer are all of the IgG class, the most dominant antibody class in plasma and non-mucosal tissues. Antibodies of the IgG class possess long half-lives in blood regulated through the neonatal Fc receptor (Reichert and Valge-Archer 2007; Roopenian and Akilesh 2007). Additionally, antibodies of the IgG class, and especially those of the IgG1 subclass, are widely thought to mediate Fc effector functions through the engagement of cellular and non-cellular components of the innate immune system such as the binding to FcγRs on immune cells including neutrophils, macrophages and NK cells (Bruggemann, Williams et al. 1987). Many studies of IgG-mediated ADCC have centered around the IgG Fc binding to FcγRIIIA on NK cells because once these cells become activated following Fc-FcR binding, it is thought they can mediate potent cytotoxic effects against tumor cells. However, NK cells have been found to poorly infiltrate solid tumors (Albertsson, Basse et al. 2003; Bowles, Wang et al. 2006), and in melanoma NK cells have been reported to be at densities significantly lower than those required *in vitro* to mediate efficient killing of melanoma cells (Balsamo, Vermi et al. 2012). Thus, antibodies of the IgG class could potentially have less potent Fc-mediated anti-tumor responses in solid tumors compared to hematological malignancies due to the distribution of immune cells.

Much recent effort has centered on engineering therapeutic antibodies to improve Fc-mediated effector functions. These strategies have included increasing the affinities of IgG antibodies to FcγRs (Kubota, Niwa et al. 2009) and engineering different antibody glycoforms (Jefferis 2012). And a few have begun to explore the therapeutic use of other immunoglobulin classes, mainly IgE and IgA, for immunotherapy (Dechant, Beyer et al. 2007; Desjarlais, Lazar et al. 2007; Karagiannis, Singer et al. 2009). Exploitation of antibody classes such as IgE for

cancer immunotherapy may allow for the engagement of alternate FcRs, such as FcεRI and CD23 (FcεRI), expressed on different immune cell populations, and the activation of these receptors in the tumor could potentially result in a potent Fc-mediated anti-tumor response. Thus, alternate classes of antibodies such as IgE targeting TAAs may have potent anti-tumor Fc-mediated effects in solid tumors and this hypothesis, proposed by Karagiannis and others, has been supported in both *in vitro* and *in vivo* models of ovarian and breast cancers (Gould, Mackay et al. 1999; Karagiannis, Wang et al. 2003; Karagiannis, Singer et al. 2009; Daniels, Leuchter et al. 2012).

An antibody of the IgE class targeting a melanoma antigen may represent a novel therapeutic modality for the treatment of melanoma. Numerous studies suggest that antibodies of the IgE class may be of interest since they have the potential to elicit Th2 immune responses in solid tumors and their use may confer some advantages over those displayed by antibodies of the IgG class. Such attributes include the lack of inhibitory receptors, higher affinity to their cognate Fc receptors, and the ability to engage many tissue resident immune cells expressing FcεRs which may be associated with tumors (Karagiannis, Josephs et al. 2012). Recent effort has focused on developing antibodies of the IgE class for the treatment of solid tumors in ovarian and breast cancer (Karagiannis, Josephs et al. 2012), but to-date this antibody class has not been evaluated for the treatment of melanoma. Thus, the discovery of novel anti-melanoma antibodies may also be achieved by developing antibodies of the IgE class targeting a known tumor antigen.

The HMW-MAA is a cell surface proteoglycan highly expressed in melanoma with low expression in normal cells, attributes that make it an attractive target for

immunotherapy (Campoli, Chang et al. 2004). HMW-MAA has been implicated in the spreading, invasion and migration of melanoma cells and murine IgG antibodies, such as the 225.28S clone, targeting this antigen have been shown to restrict tumor growth in mouse models of melanoma (Chang, Campoli et al. 2004; Hafner, Breiteneder et al. 2005). In the clinical setting, passive immunotherapeutic approaches employing murine anti-idiotypic antibodies to generate host antibody responses to HMW-MAA have been met with some response in patients (Mittelman, Chen et al. 1990; Mittelman, Chen et al. 1992). Immunotherapeutic strategies targeting the HMW-MAA have focused on the use of these anti-idiotype murine antibodies (Mittelman, Chen et al. 1990; Mittelman, Chen et al. 1992), antigenic mimitopes (Wagner, Hafner et al. 2005; Luo, Ko et al. 2006), full length murine antibodies (Hafner, Breiteneder et al. 2005), and human scFv antibody (Wang, Katayama et al. 2011) recognizing this antigen. The clinical utility of recombinant antibodies with a human Fc region such as chimeric/humanized monoclonal antibodies targeting HMW-MAA has not yet been demonstrated in melanoma. It is expected that these antibodies may have direct effects on the function of this antigen, but the efficacy of such antibodies in the clinic may also originate from immunological mechanisms of action, which could include their ability to engage immune cells to specifically target melanoma cells. In light of the importance of Fc-mediated mechanisms of action in therapeutic anti-tumor responses and the emerging evidence that antibodies of different classes may activate immune responses against cancer, the mechanisms of action of IgG and IgE antibodies recognizing HMW-MAA merit further dissection in the appraisal of these molecules as passive immunotherapies for the treatment of melanoma.

The aim of this chapter is to evaluate novel monoclonal antibodies recognizing melanoma cell surface antigens and specifically to:

1. Identify and characterize a melanoma-reactive monoclonal antibody derived from a patient.
2. Evaluate the *in vitro* function of human Fc bearing IgG and IgE antibodies targeting the HMW-MAA to mediate both Fab and Fc effector mechanisms.

5.2 Patient-derived Monoclonal Antibodies

The successful establishment of antibody-secreting B cell cultures from individuals with melanoma and development of screening methods (Chapter 3) in conjunction with the findings of a prevalent antibody response to melanoma cells among patients with melanoma (Chapter 4) supported the feasibility of the approach described herein to identify melanoma-specific antibodies from patients.

5.2.1 Strategies for Discovering Anti-melanoma Antibodies from Clinical Specimens

A strategy leading to the possible discovery of melanoma-specific antibodies from peripheral blood B cells (Figure 5.1) was devised based on the findings presented Chapter 3. Following the isolation of B cells, *ex vivo* antibody-secreting B cell cultures were established from individuals and cell culture supernatants were evaluated for reactivity to melanoma cells using the cell based-ELISA (Chapter 2,

Section 2.7.5). The reactivity of antibodies in these cultures to melanoma cells was measured relative to the reactivity of a non-specific human IgG antibody (fold increase) which allowed for the comparison of the reactivity of cultures across multiple cell lines (detailed in Chapter 2, Section 2.8.1). Depending on the amount of available supernatant for a given culture, the reactivity of antibodies in cultures was evaluated across multiple melanoma cell lines and primary human melanocytes (Figure 5.2). Cultures with high reactivity to one or more melanoma cell lines relative to melanocytes would then be selected for expansion. A limited number of cultures were selected for further subcloning and the derivation of monoclonal cultures. For example, following the screening of B cell cultures (n=60) derived from one patient (M120) against three melanoma cells lines and melanocytes, culture wells such as B6, F10 and G11 would be selected based on reactivity to melanoma cell lines relative to melanocytes and would be further maintained in culture for subcloning (Figure 5.2). Cultures which displayed similar reactivity among melanoma cells and melanocytes, such as culture G9 would not be selected for further expansion and subcloning (Figure 5.2).

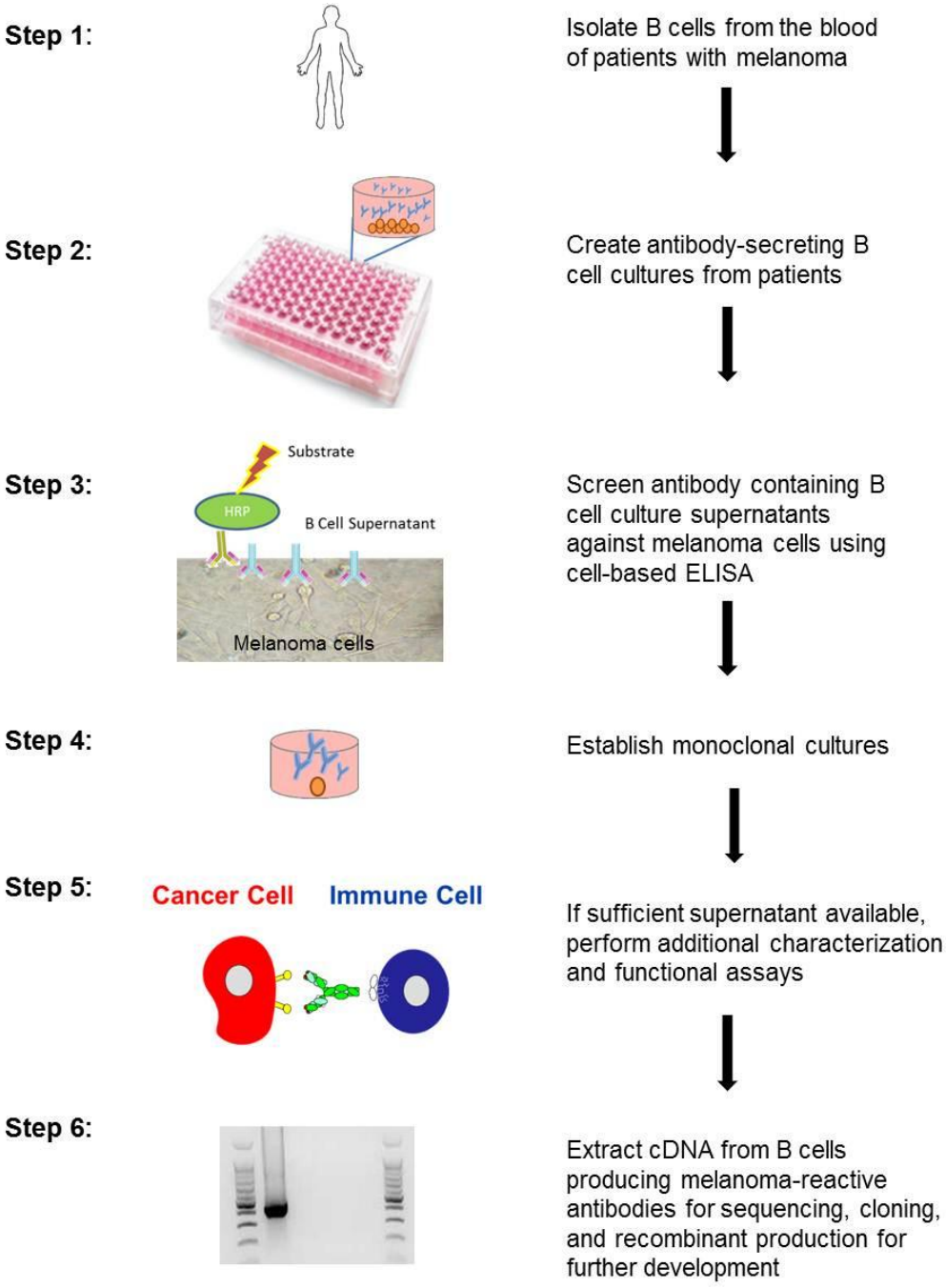


Figure 5.1 Schematic of the process for discovering melanoma-reactive antibodies from patients. Diagram highlighting the process devised to identify novel monoclonal antibodies from patients' B cells for the production of recombinant antibodies for further characterization.

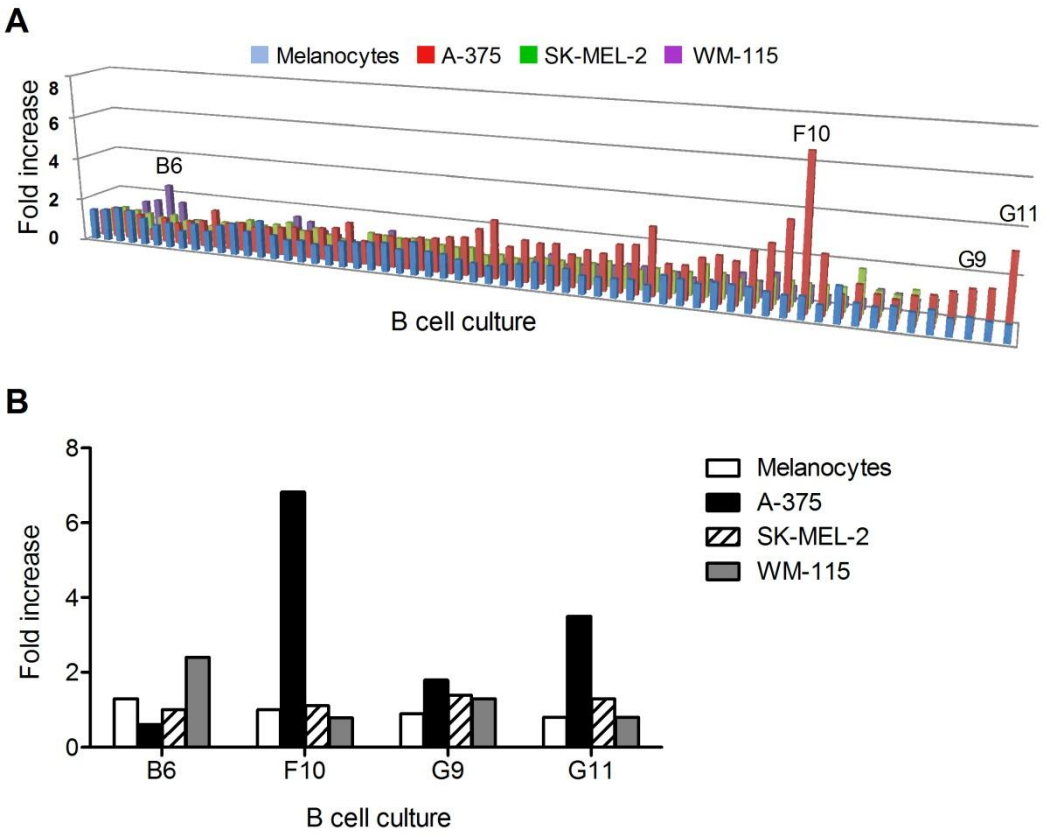


Figure 5.2 Example of cell-based ELISA screening results for B cell cultures derived from one patient showing reactivity against human melanoma cells and melanocytes. (A) Supernatant from a 96 well plate containing 60 B cell culture wells was screened against melanocytes and three melanoma cell lines using the cell-based ELISA. (B) Selected clones from one individual (from A) illustrate the different degrees of binding of supernatants to melanoma cells and melanocytes. Fold change values represent absorbance values of each well divided by the absorbance value of a non-specific IgG antibody tested against the same cell line.

B cell cultures from multiple patients were selected based on their high reactivity to melanoma cells relative to their reactivity to normal cells such as melanocytes. Promising cultures from multiple individuals were evaluated for reactivity using the cell-based ELISA against multiple cell lines. For example, the reactivity of antibody cultures from multiple patients was assessed against four melanoma cell lines and melanocytes, and these results illustrate the variation of the reactivity of cultures to different melanoma cell lines relative to melanocytes (Figure 5.3). Of these antibody-producing cultures, some displayed less overall reactivity across melanoma cell lines than others, such as culture M130_1E5 where reactivity was only higher for one (A-375) of the four melanoma cell lines tested relative to melanocytes (Figure 5.3). Other cultures, such as M133_1D4, displayed high reactivity to three out of the four melanoma cell lines evaluated relative to melanocytes (Figure 5.3), and represented the most promising antibody candidates. Antibodies reactive to multiple cell lines, with each cell line derived from a separate individual, signified promising candidates, since these antibodies bound to antigens expressed across individuals rather than just autologous cells. Antibodies with reactivity to multiple melanoma cell lines could potentially represent reactivity against novel antigens highly expressed across individuals with melanoma, thus making the particular clone an attractive candidate to be tested as a possible immunotherapeutic with potentially widespread future use.

Antibodies with lower reactivity to melanoma cells, such as those that were found to bind to only one of the melanoma cell lines examined, could also represent promising novel monoclonal antibodies with therapeutic potential. While this low reactivity could reflect restricted expression of the antigen in the population, these antibodies may still hold some promise in the treatment of aggressive sub-types of

cancer for which there are no or limited available treatments. An example of such an antibody is Trastuzumab, which is effective in prolonging survival in the context of breast cancer. Trastuzumab, while targeting Her2/neu, an antigen expressed only in a relatively small proportion (20-30%) of breast cancers, still constitutes an effective therapeutic option for the subset of patients expressing the Her2/neu antigen (Slamon, Godolphin et al. 1989; Vogel, Cobleigh et al. 2002). Thus, patient-derived antibodies with reactivity to at least one melanoma cell line retain possible therapeutic potential.

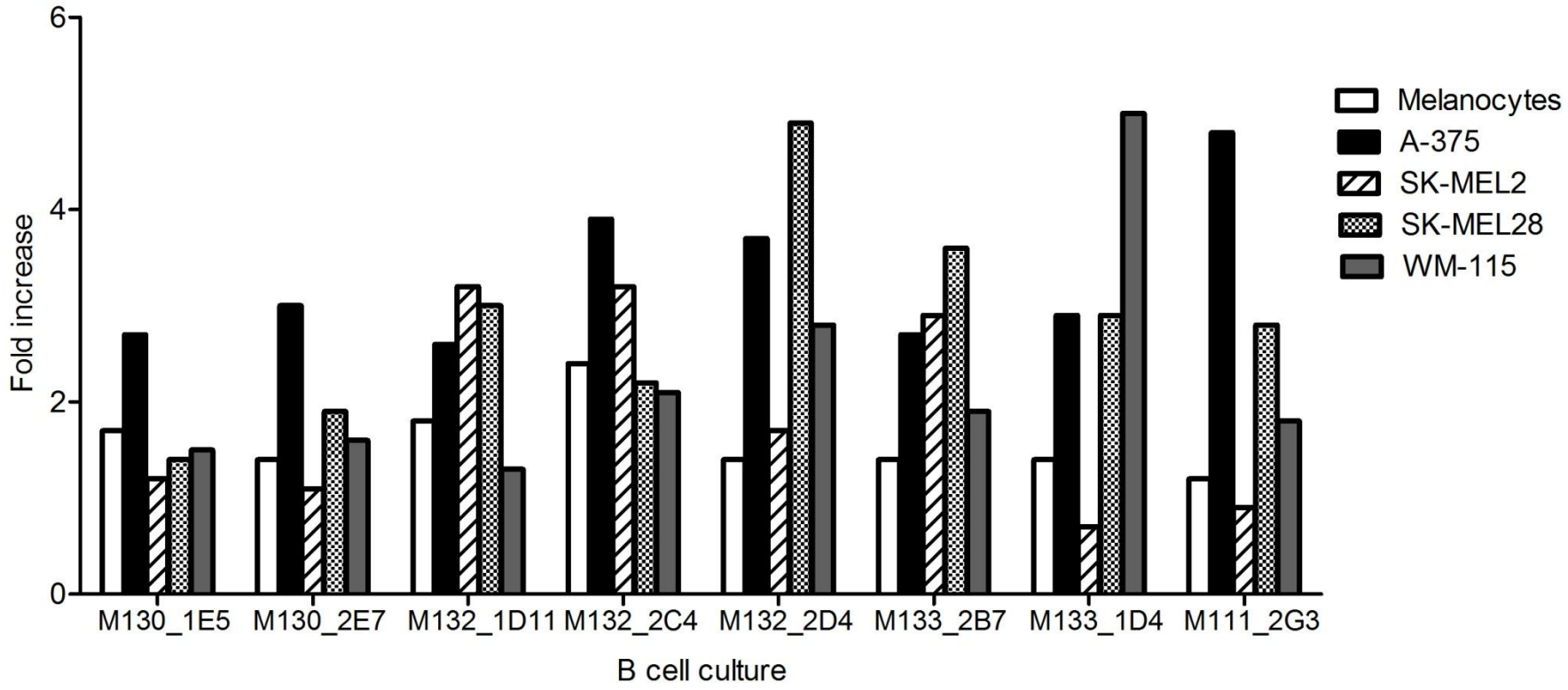


Figure 5.3 Screening of selected B cell cultures from four patients against multiple melanoma cell lines compared to melanocytes. B cell cultures derived from four different patients (M130, M132, M133, and M111) were screened against four melanoma cell lines and melanocytes using a cell-based ELISA. Fold increase values represent optical density values of each well divided by the optical density of a non-specific IgG antibody tested against the same cell line. Cultures were selected to illustrate the variation of binding to different **melanoma** cell lines compared to melanocytes.

After selecting promising antibody cultures using the criteria above, monoclonal B cell cultures were then derived by serial dilution (detailed in Chapter 2, Section 2.8.2). Antibodies from these monoclonal cultures were then further evaluated for reactivity to multiple melanoma cell lines and melanocytes either by the cell-based ELISA or by flow cytometric analyses. Further evaluations of reactivity were conducted: (1) to confirm the reactivity of the clone after serial dilution and (2) to expand on initial evaluations of specificity. One monoclonal antibody, M111_2G3, was selected from a panel of antibodies (Figure 5.3) and was then evaluated for specificity to additional melanoma cells along with other normal cells, such as dermal fibroblasts, using flow cytometry. This M111_2G3 antibody clone showed reactivity to two melanoma metastatic cell lines, A-375 and SK-MEL-28, but not to primary human skin cells, such as dermal fibroblasts or melanocytes derived from healthy volunteers (Figure 5.4). These flow cytometric analyses of the monoclonal culture showed concordance with the initial screening results obtained from the originating cultures utilizing the cell-based ELISA, where reactivity was also measured against the A-375 and SK-MEL-28 cell lines but not melanocytes (Figure 5.3). In summary, these results demonstrate the presence of an antibody clone with reactivity to melanoma cells and not melanocytes following the creation of monoclonal cultures from *ex vivo* patient B cells.

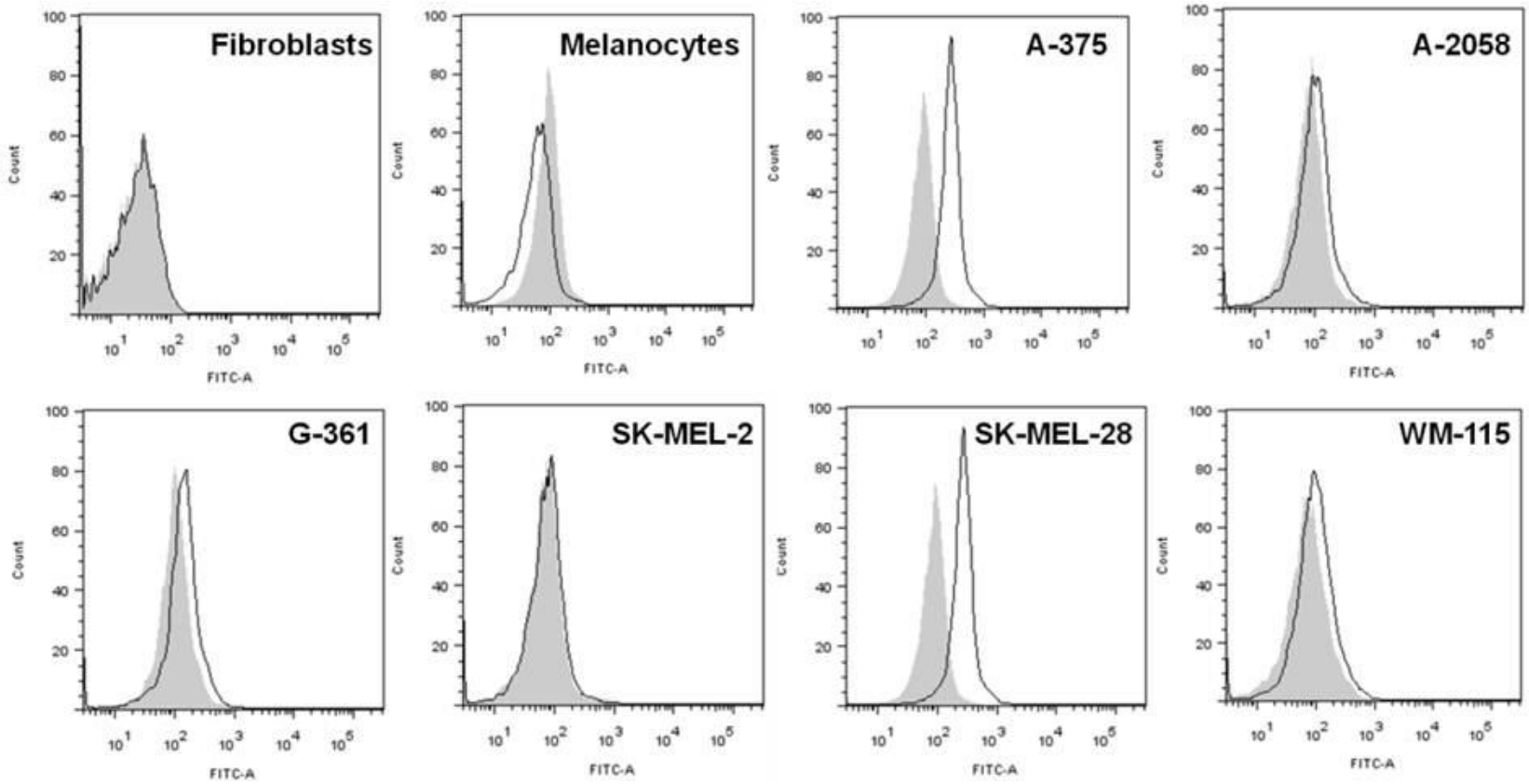


Figure 5.4 Evaluation of the binding of the patient-derived monoclonal antibody M111_2G3 to normal skin cells and melanoma cell lines. Antibody clone M111_2G3 was evaluated for binding to 6 melanoma cell lines and also to primary fibroblasts and melanocytes by flow cytometry. Binding of M111_2G3 clone (black line) to each cell line was evaluated compared to a non-specific human IgG isotype control (shaded grey).

Insufficient amounts of antibodies posed a major limitation of this method for many of the clones derived. After 2 to 3 months in culture, B cell division ceased and frequent contaminations in the laboratory proved particularly challenging in establishing monoclonal cultures. This meant that some of the most promising clones, such as M132_2D4 and M133_1D4 (Figure 5.4), were lost due to contamination following the establishment of monoclonal cultures. This limitation was overcome in some cases by extracting ribonucleic acid (RNA) from cells prior to their contamination or decrease in proliferation. RNA from these B cells could be sequenced to obtain variable region sequences and then used to engineer recombinant forms of the antibodies. An alternative way to circumvent these limitations of cell proliferation and contamination in the future would be to identify single B cells with reactivity to melanoma cells by tetramers or other fluorescent detection technologies in order to select single tumor-reactive B cells and produce antibodies of interest in recombinant form (Tiller, Meffre et al. 2008). Such solutions may yield additional melanoma-reactive monoclonal antibodies but would require the time consuming work of identifying antibody reactivity from a single B cell, cloning, expression, and production of ample amounts of antibodies for further characterization.

In summary, a process for discovering novel antibodies by harnessing the natural antibody response to cancer has been described. This method yielded the monoclonal antibody M111_2G3 which demonstrated specificity to melanoma cells and not melanocytes. Next, the potential functional properties of this anti-tumor antibody will be explored utilizing *in vitro* cytotoxicity assays.

5.2.2 Characterization of Patient-derived Monoclonal Antibodies

While the presence of B cells producing tumor-reactive antibodies has been described in the serum and tumor lesions from patients (Kirkwood and Robinson 1990; Punt, Barbuto et al. 1994), the capacity of such antibodies to elicit an anti-tumor response has not yet been characterized. Using a melanoma-specific monoclonal antibody derived from a patient diagnosed with Stage III melanoma, M111_2G3, the ability of this patient-derived antibody to mediate cytotoxicity was evaluated *in vitro* using A-375 melanoma cells and human monocytic effector cells. The addition of immune effector cells in this assay allowed for assessments of ADCC using the limited amount of antibody available. These evaluations were performed alongside another antibody clone, M111_2D10. Clone M111_2D_10 was a monoclonal antibody produced from the same individual and manner as M111_2G3, but was found to have no significant reactivity to A-375 cells compared to melanocytes (Figure 5.5). This antibody therefore served as a negative control for the functional assays that followed, which evaluated the cytotoxic potential of patient-derived antibodies against melanoma cells.

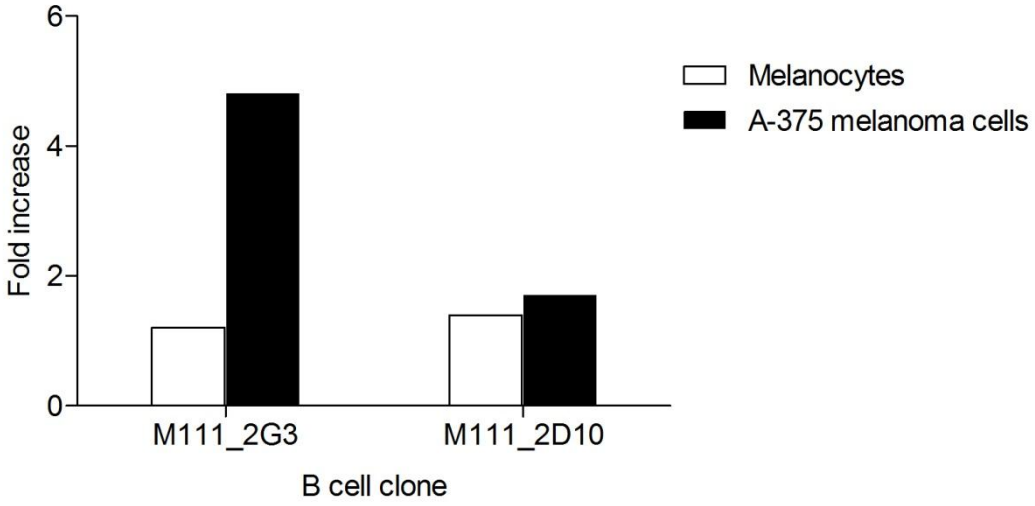


Figure 5.5 Reactivity of two monoclonal antibodies derived from patient M111 to melanocytes and melanoma cells. Two monoclonal antibodies, M111_2G3 and M111_2D10, were evaluated for reactivity to A-375 melanoma using the cell-based ELISA. Fold increase values represent absorbance values above the mean absorbance of the non-specific IgG control antibody. These antibodies were evaluated for their potential cytotoxicity *in vitro* against A-375 melanoma cells.

The ability of patient-derived antibodies to mediate cytotoxicity against melanoma cells was assessed using a real-time live-dead cell cytotoxicity assay (described in detail in Chapter 2, Section 2.10.3). These assays required low amounts of antibodies and were therefore selected to perform functional characterizations using limited amount of antibody available. In these experiments, U-937 human monocytic cells were used as effector cells since they are known to express FcγRs (Anderson and Abraham 1980) and have been shown by others to have effector function that correlates with *in vivo* efficacy against tumors using primary human monocytes (Karagiannis, Bracher et al. 2007; Karagiannis, Bracher et al. 2008).

The potential of the patient-derived melanoma-specific antibody M111_2G3 to mediate cytotoxic activity against A-375 tumor cells was then assessed and compared to the activities of the non-specific control monoclonal antibody M111_2D10. Employing the live-dead cell cytotoxicity imaging assays, antibody mediated cellular cytotoxicity could be visualized for live melanoma cells (green) incubated with monocytic cells (blue) when combined with the patient derived melanoma-specific monoclonal antibody (M111_2G3) over a period of two hours (Figure 5.6, top panel). In these assays, the loss of viability of live tumor cells incubated with effector cells and the antibody clone M111_2G3 could be seen by the loss of green fluorescence in tumor cells while the loss of membrane integrity was confirmed by the incorporation of red dye (ethidium homodimer-1) into the DNA of dead cells over a two hour period at 37˚C. Increased amounts of tumor cell death were evident after 2 hours of incubation with melanoma specific antibody M111_2G3 (Figure 5.6, top panel), compared to the non-melanoma specific antibody control M111_2D10 (Figure 5.6, bottom panel). Monocyte cell viability was maintained throughout the assay and was similar between cultures treated with either the M111_2G3 antibody or the M111_2D10 non-specific control (Figure 5.6).

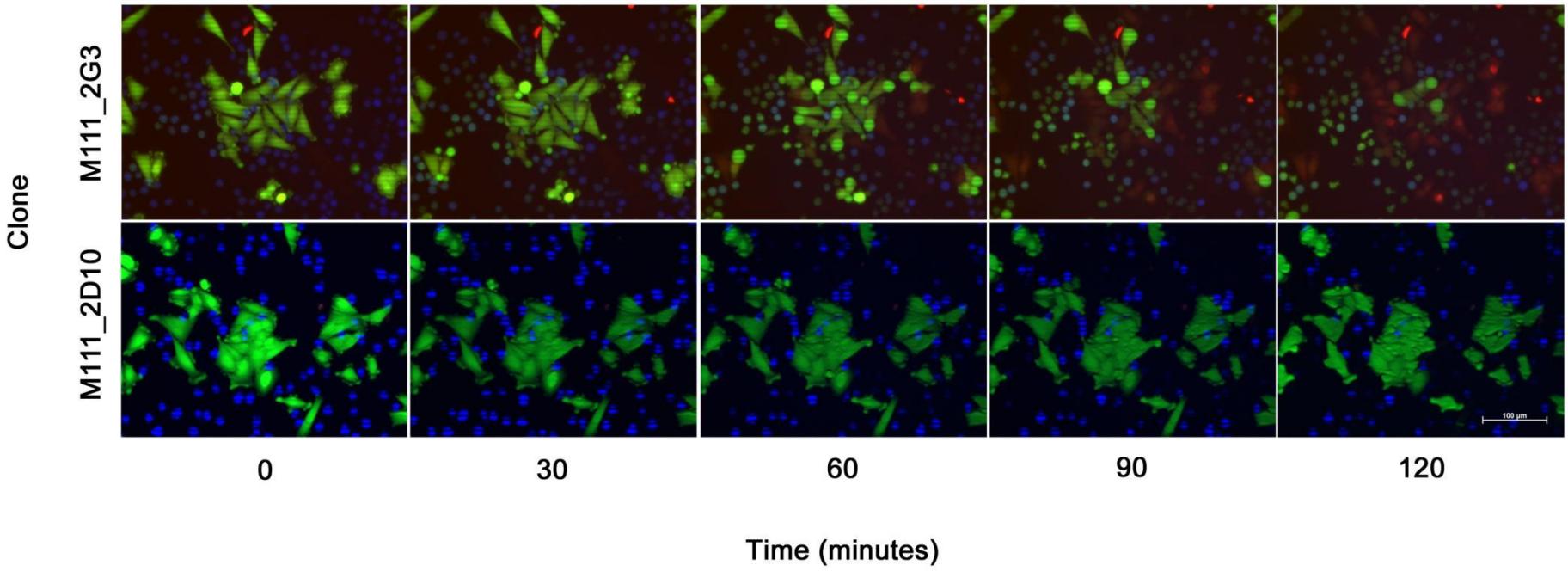


Figure 5.6 Evaluation of a patient-derived melanoma-specific antibody to mediate tumor cell cytotoxicity. Two monoclonal antibodies from patients were evaluated *in vitro* for cytotoxic effects using a live cell imaging assay. Clone M111_2G3 bound to A-375 melanoma cells and clone M111_2D10, derived from the same patient as the tumor-specific clone M111_2G3, had no specificity to A-375 melanoma cells and thus served as a negative control antibody. Fluorescent images of the live cell cytotoxicity assays are shown at 30 minute intervals. Live calcein AM labeled A-375 melanoma cells (green) were incubated with M111_2G3 (top) or M111_2D10 (bottom) antibodies and U-937 monocytic effector cells (blue). Cell death was detected by the loss of calcein AM and confirmed by the incorporation of ethidium homodimer-1incorporation into cells (red). Magnification 20X, Scale bar: 100µm.

The cytotoxic effects of antibodies against melanoma cells were quantitated by measuring the fluorescence intensity of melanoma cells in these assays. Live melanoma cell fluorescence intensity was reduced to 18% of the original intensity values when cells were incubated with the tumor specific antibody M111_2G3, whereas cells incubated with the non-specific control M111_2D10 antibody demonstrated reduced fluorescence to only 64% of the original intensity values relative to the start of the assay (Figure 5.7, top). Loss of fluorescence observed in the control sample may be attributed to a combination of background loss of viability in culture conditions, non-specific cytotoxicity triggered by human effector cells armed with Fc receptor-bound antibodies, and also photo bleaching effects brought about from frequent capturing of images during assay setup. When tumor cell viability was assessed by measuring the incorporation of ethidium homodimer-1 by tumor cells for each assay condition, a statistically significant decrease ($P<0.001$) in tumor cell viability was observed when melanoma cells and effector cells were incubated with a patient-derived melanoma-specific antibody (18%, SD=9.5%) compared to the non-specific antibody M111_2D10 (95%, SD=5.8%) (Figure 5.7). The loss of fluorescence of live melanoma cells in combination with the uptake of ethidium homodimer-1 demonstrate that an antibody derived from a patient in the presence of immune effector cells was capable of mediating cellular cytotoxicity *in vitro* against melanoma cells.

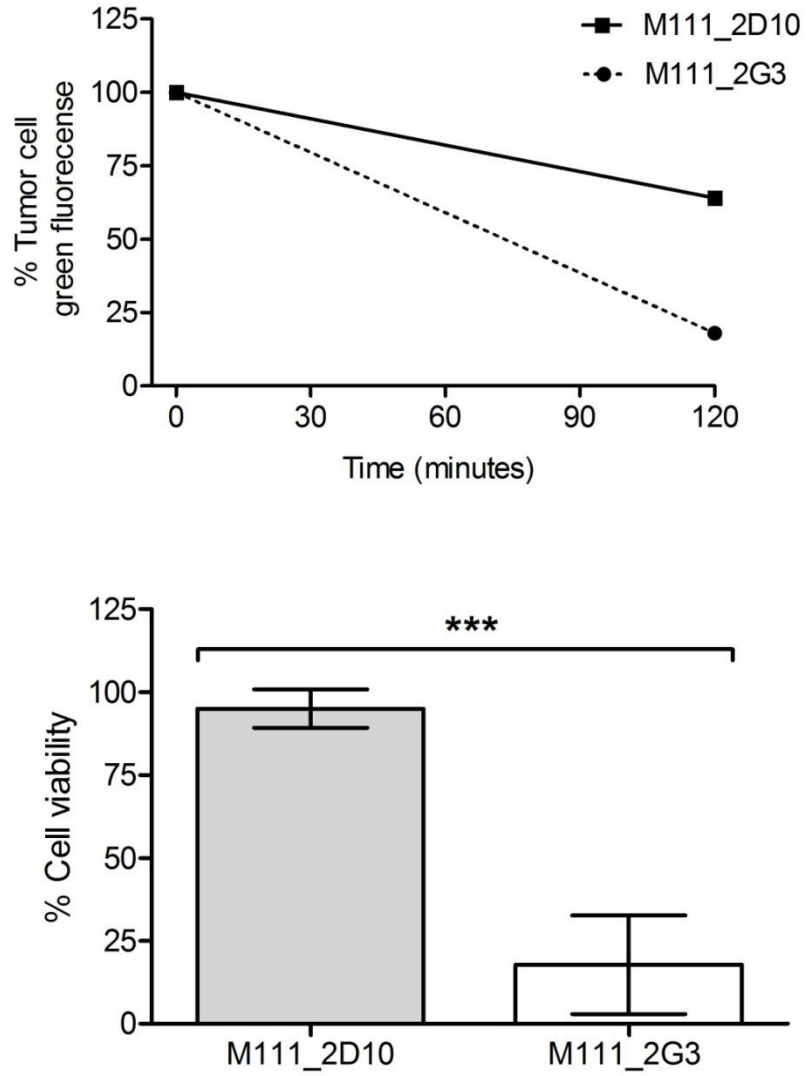
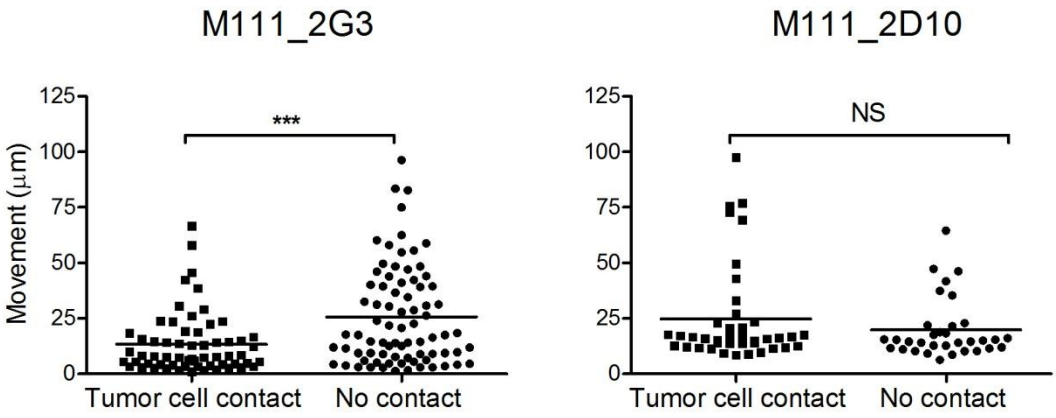


Figure 5.7 Quantitative analysis of antibody-mediated cellular cytotoxicity against tumor cells by patient-derived monoclonal antibodies. Antibody-mediated cellular cytotoxicity of a patient-derived melanoma-reactive antibody, Clone M111_2G3, was compared to an antibody clone with no reactivity to the same melanoma cells, Clone M111_2D10, when cultured in the presence of tumor cells and monocytic effector cells. A-375 cells were stained with calcium AM (green) and cell death was measured by loss of green fluorescence (top) and the incorporation of ethidium homodimer-1 (red) into tumor cells (bottom). Mean and SD (bottom figure) were calculated for four separate regions within the frame, while total green fluorescence was measured for the entire frame. *** $P<0.001$. P values were calculated using the two-sided Student's t test.

To further evaluate if melanoma cell death was potentially mediated by the engagement and activation of FcγRs on immune effector cells, the movement of these effector cells in relation to melanoma cells was tracked in these experiments. For cells incubated with the melanoma-specific antibody M111_2G3, there was a significant (*P*=0.002) reduction in the movement of effector cells once in contact with melanoma cells (13 μm, 95% CI=10 to 17 μm) compared to effector cells not in contact with melanoma cells (25 μm, 95% CI= 18 to 31 μm) (Figure 5.8, left). Further analysis showed that for melanoma cells incubated with the non-specific M111_2D10 clone, no difference was seen in the movement of effector cells in contact with tumor cells compared to effector cells not in contact with tumor cells (Figure 5.8, right). Thus a significant reduction in the movement of effector cells was only seen once effector cells were in contact with tumor-specific antibody coated tumor cells. These observations provide further evidence that antibody-mediated engagement of effector cells may be necessary for cell death, suggesting that ADCC may be a mechanism by which this patient-derived antibody could function against melanoma cells.

A



B

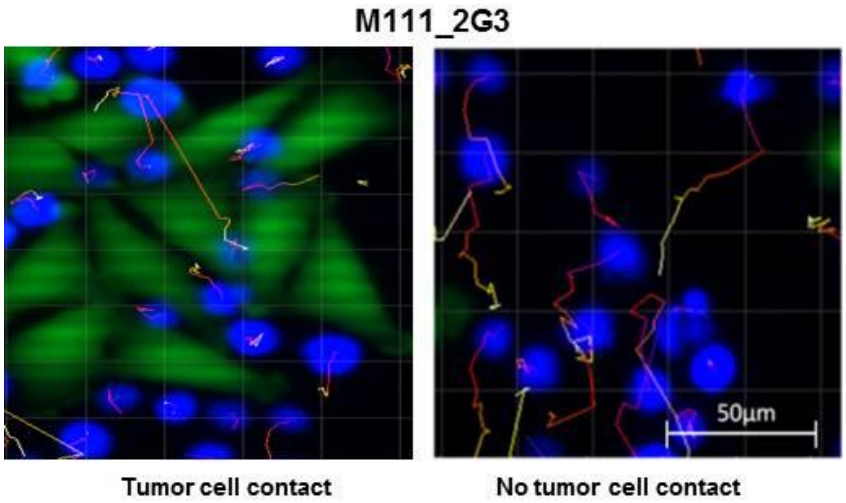


Figure 5.8 Movement of effector cells incubated with patient-derived melanoma reactive and non-reactive antibodies and melanoma tumor cells. (A) Movement of monocytic effector cells, U-937, was tracked and measured over two hours and compared between cells in contact with tumor cells and cells not in contact with tumor cells. This was done for the M111_2G3 melanoma-reactive clone (left) and non-reactive clone M111_2D10 (right). (B) Images of U-937 cell movement in tumor cell cultures treated with M111_2G3, tracked for cells in contact (left) and cells not in contact with tumor cells (right). Movement is indicated by tracking lines (red to yellow) from the original position of U-937 cells at t = 0 to t = 2 hours (magnification 20x, Scale bar: 50 µm). *** $P<0.001$. P values were calculated using the two-sided Students t test. Data courtesy of Dr. Panagiotis Karagiannis, KCL.

Further functional assessments of this M111_2G3 antibody clone, along with identification of the antigen it recognizes, were limited by the amount of antibodies produced from B cell cultures. To overcome this limitation, the extraction of RNA from these cells was attempted in order to obtain variable region sequences to be used to engineer a recombinant form of this antibody clone. Unfortunately, this was not achieved for clone M111_2G3 because RNA quality was poor from the very small number of cells (10^3) remaining in the culture which were subsequently frozen and possibly suffered a loss of cell integrity and viability through the freezing process. However, this limitation was later overcome for other B cell clones by directly placing live cells in RNAlater® Tissue Collection: RNA Stabilization Solution (Invitrogen). Although it was not possible to obtain the sequence of clone M111_2G3 variable regions within the timeframe of this project, the sequence of another clone, M80_1F2, derived using the same methodology from another patient (M80, Table 4.1) and displaying reactivity to melanoma cells, was obtained (Figure 5.9). These heavy and light chain variable regions could then be inserted into vectors containing the sequences for human IgG1 Fc regions. Transfection of competent cells with these vectors would then result in the recombinant production of antibodies in sufficient quantities to perform broader characterization including further functional assays such as those evaluating Fab and Fc-mediated mechanisms of action.

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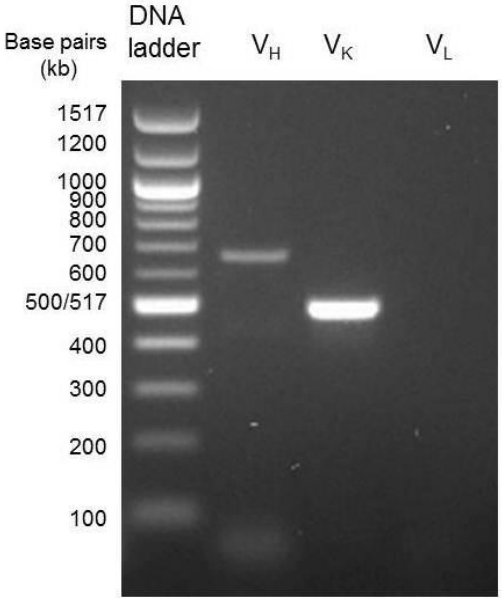

B

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TCCTGTGCAGCCTCTGGATTCACCTTTAGGCACTATGCCATCAGTTGGGTCCGCCAGGCTC
CAGGGAAGGGGCTGGAGTGGGTCTCAGGTCTGAGTGGTAGTGGAAATAGGACATACTAC
GCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCGAGAACACGCTGTTT
TTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCGAAAGATCGC
CGAGTGGGAGCTACCTTCGTCTTTGACTCCTGGGGCCAGGGAACCCTGGTCACCGTCTCCC
CAG

C

ATGACCCAGACTCCATCCACCCTGTCTGCATCTGTGGGAGACAGAGTCACCATCACTTGCC
GGGCAAGTCAGAACATTACCACCTATTTCAATTGGTATCAGCAAAAACCAGGGAAAGCCC
CTAAACTCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAAGGTTCAGTGG
CAGTGGATCTGGGACAGATTTCACTCTCACCATCACCAGTCTGCAACCTGAAGATTTTGCA
ACTTACTACTGTCAACAGAGGGGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACG
A

Figure 5.9 Variable region nucleic acid sequences and nested PCR for a patient-derived melanoma-reactive antibody clone. (A) Nested PCR amplification of heavy and light chain variable regions for antibody clone M80_1F2 derived from a patient. V_H= variable region heavy chain; V_K= variable region kappa chain; V_L= variable region lambda chain. 1 kilobase DNA ladder was used (New England Biolabs) on 1.5% agarose gel stained with ethidium bromide. (B) Heavy chain variable region sequence for M80_1F2. (C) Kappa light chain variable region sequence for M80_1F2. Data courtesy of Mr. Tihomir Dodev, KCL.

In summary, a translational approach to discovering novel antibodies from melanoma patient memory B cells has been described. This approach yielded a melanoma-specific monoclonal antibody, M111_2G3, which has shown some potential *in vitro* to mediate tumor cell killing. While further characterization of this antibody was limited by the amount of antibody available, limitations to this approach could be overcome in the future by RNA extraction from B cells and recombinant expression of such antibodies, as demonstrated here for a different patient-derived clone. Thus, harnessing the cancer-specific antibody repertoire of melanoma patients offers an alternate strategy for the discovery of novel monoclonal antibodies with potential cytotoxic activity. Next, further assessments of antibody function will be explored using engineered antibodies against a known melanoma antigen. Such assessments could also prove useful in the future to elucidate possible mechanisms of action of new patient-derived antibody clones.

5.3 Evaluations of IgG1 and IgE Antibodies Targeting a Melanoma Antigen

The HMW-MAA is a large molecule comprised of a 450 kDa chondroitin sulfate proteoglycan and a 250 kDa core glycoprotein, and several antibodies have been generated against different regions of this molecule (Ziai, Imberti et al. 1987). The 225.28S antibody clone recognizes an epitope of HMW-MAA residing on the plasma membrane (Wilson, Imai et al. 1981) and from linear peptide mapping studies it is has been suggested that the epitope resides in the 250 kDa core

glycoprotein in a region free of internal/flanking glycosylation sites and low proteolytic sites (Mittelman, Tiwari et al. 2004). Since heavy glycosylation or proteolysis could prevent antibody recognition of its epitope, protein regions lacking these features make ideal antibody targets. The 225.28S antibody was selected for further study because of its epitope location on the cell surface along with favorable preclinical efficacy data from the murine version of the antibody in animal models (Ghose, Ferrone et al. 1991; Hafner, Breiteneder et al. 2005). The antibody clone was also selected based on promising clinical results from a study involving 19 patients using active immunotherapy targeting its epitope, where one patient was observed to have a complete remission and 3 patients had partial responses (Mittelman, Chen et al. 1990).

More specifically, the 225.28S anti-HMW-MAA antibody clone (referred to as HMW-MAA antibodies hereafter) poses an attractive immunotherapeutic option for the treatment of melanoma since it has previously demonstrated some preclinical promise. Preclinical studies with murine HMW-MAA antibodies have highlighted the potential of these antibodies to inhibit the growth and migration of melanoma cells both *in vitro* and in a SCID mouse xenotransplantation model of melanoma (Hafner, Breiteneder et al. 2005). Because these studies employed a murine antibody, any potential Fc-mediated immunological mechanisms of action in either *in vitro* or *in vivo* models could not be evaluated in the context of the capacity of such antibodies to engage human immune cells. Previously, the murine HMW-MAA antibody had been reported to be a poor mediator of CDC and ADCC in mice (Imai, Pellegrino et al. 1982); however, these results, dating back 30 years, may not necessarily correlate to human Fc-mediated effector function. Further study of the ability of these antibodies to engage human immune effector cells is

warranted through the use of sophisticated methodologies available at the present time, such as those used to evaluate ADCC and ADCP (Bracher, Gould et al. 2007), to possibly provide insights into the Fc effector functions of these molecules. Clinical development of HMW-MAA antibodies has been hindered by their lack of human forms, and the generation of HMW-MAA antibodies with human Fc regions for further preclinical evaluation may provide insights into the potential clinical utility of this antibody clone for passive immunotherapy.

5.3.1 Characterization of Engineered HMW-MAA Antibodies of the IgG1 and IgE Class

HMW-MAA antibodies were designed with human Fc regions of the IgG1 and IgE classes using previously published variable region sequences (Neri, Natali et al. 1996). Using these amino acid sequences, cDNA encoding for the variable regions of the antibody was synthesized (Gene Art AG) and cloned into a vector containing the cDNA encoding for either the epsilon heavy chain of IgE or the gamma heavy chain of IgG1, along with another vector containing the cDNA encoding for the human kappa light chain constant region. All of these constant regions were synthesized from the cDNA of human B cells. DNA from the vectors was produced using HiSpeed Plasmid Maxi Kit® (Qiagen®), transfected into human embryonic kidney 293 cells which were harvested after 2-4 weeks in culture, and antibodies were purified from cell culture supernatant using affinity chromatography as described previously (Gould, Mackay et al. 1999; Karagiannis, Singer et al. 2009). The construction, production, and biochemical characterization of HMW-MAA

antibodies of the IgG and IgE class were performed by Mr. Tihomir Dodev, KCL, who kindly provided antibodies for the functional studies described below.

HMW-MAA antibodies were characterized following antibody cloning, expression and purification to confirm the identity and purity of these IgG and IgE antibodies. By us sing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the molecular size of these antibodies could be assessed relative to the molecular markers (protein ladder) of a known molecular size and compared to IgG and IgE isotype control antibodies. By SDS-PAGE, under reducing conditions, the expected molecular masses of the heavy and light chains for HMW-MAA IgG and IgE were observed to be similar to the anti-FRα MOv18 IgG1 and IgE chimeric antibody isotype controls (Figure 5.10A). Similarly, the molecular masses for whole Ig molecules under non-reducing conditions evaluated by SDS-PAGE for both HMW-MAA IgG and IgE antibodies were of expected size and similar to their corresponding isotype control antibodies (Figure 5.10A). These results confirm the production of proteins which are the same size as IgG and IgE molecules. Next the presence of any impurities, degradation products, or aggregates in the antibody preparations was evaluated by size exclusion chromatography. The absence of significant aggregation or degradation of antibodies along with the absence of impurities in antibody preparations were confirmed by size exclusion chromatography evaluations, since the elution of antibodies was seen at the same time as their respective isotype control antibodies without any additional products (or peaks) detected (Figure 5.10B). No additional products before or after the elution of the antibody were detected, which, if found, would have indicated antibody degradation or aggregation, respectively (Figure 5.10B).

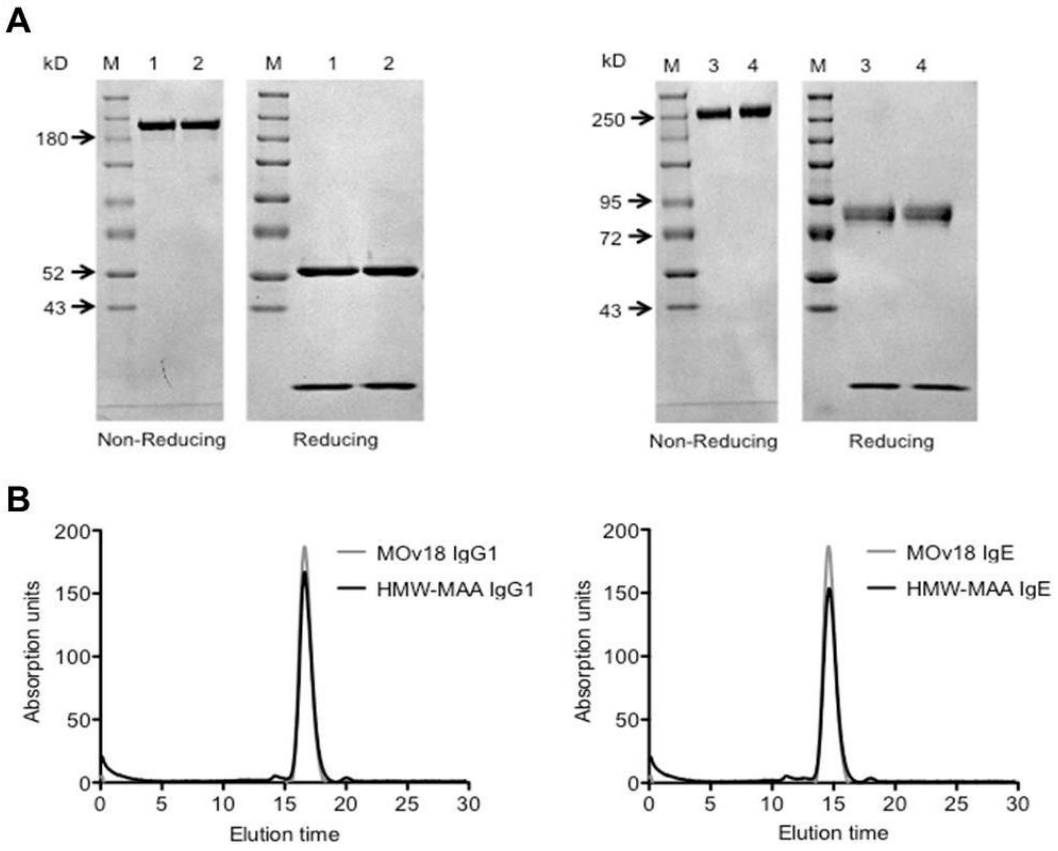


Figure 5.10 Characterization of engineered antibodies of the IgG and IgE classes targeting HMW-MAA. (A) SDS-PAGE analysis of purified HMW-MAA IgG1 (l) and control MOv18IgG1 (2) (left) under non-reducing and reducing conditions and their isotype HMW-MAA IgE (3) and control MOv18IgE (4) versions (right), along with (M) Spectra™ Multicolor High Range Protein Ladder (Fermentas) visualized by Coomassie staining. (B) Size exclusion chromatography analysis of HMW-MAA antibodies and MOv18 antibody isotype controls. Figure courtesy of Mr. Tihomir Dodev, KCL.

Next, the Fab and Fc specificities of engineered HMW-MAA antibodies were evaluated. Had recombinant forms of the antigen been available, antigen binding and estimations of affinity could have been assessed by surface plasma resonance or ELISA. However, due to the lack of recombinant antigen, the binding of antibody to antigen was assessed using cells known to express the HMW-MAA antigen (A-375) and also cells known to lack expression of HMW-MAA (melanocytes). The binding of HMW-MAA antibodies of the IgG and IgE classes to A-375 cells but not to melanocytes was observed by flow cytometry (Figure 5.11). Additionally, the binding of the 225.28S HMW-MAA IgG antibody to A-375 cells by flow cytometry was similar to the binding of another murine HMW-MAA monoclonal antibody clone (Clone LHM2, Invitrogen) generated following the immunization of mice with a crude cell extract of A-375 cells (see Chapter 3, Figure 3.8), which was consistent with the specificity of HMW-MAA antibodies to melanoma cells. Both IgG and IgE HMW-MAA antibodies were also found to bind to monocyte cells isolated from a healthy volunteer (Figure 5.12), known to express FcγRs and FcεRs, by flow cytometry. These results display the production of antibodies of the IgG and IgE classes with specificity to the HMW-MAA antigen expressed on melanoma cells with the ability to bind to Fc receptor bearing immune cells such as human monocytes.

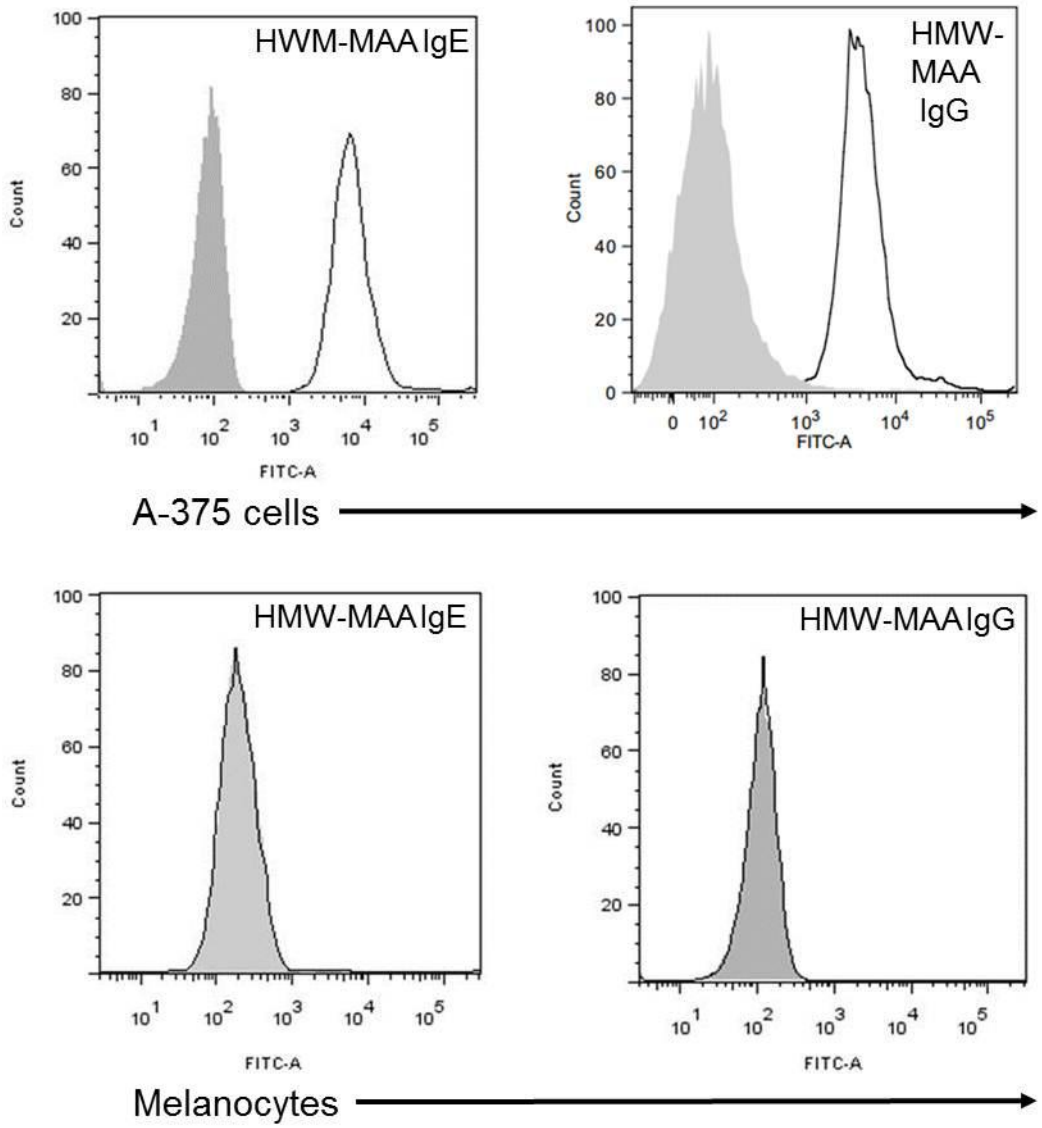


Figure 5.11 Binding of HMW-MAA IgG and IgE to A-375 melanoma cells and melanocytes. The binding of 225.28S HMW-MA chimeric antibodies, depicted with a black line, to A-375 melanoma cells (top) and melanocytes (bottom) was evaluated by flow cytometry. Non-specific isotype controls, MOv18 IgG and IgE, are shown in shaded grey.

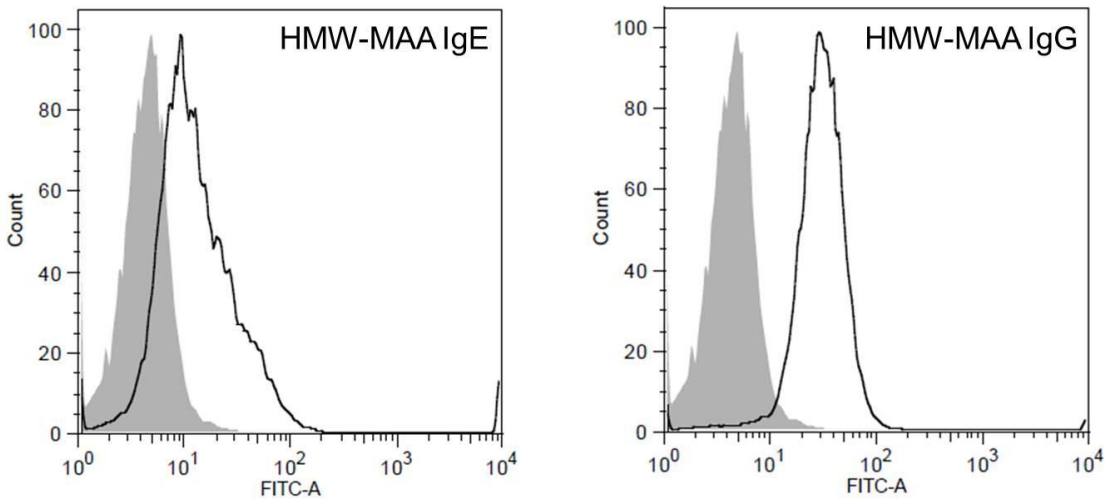


Figure 5.12 Binding of HMW-MAA IgG and IgE to human monocytes. The binding of chimeric 225.28S HMW-MAA antibodies, depicted with a black line, to human monocytes isolated from a healthy volunteer was evaluated by flow cytometry. Non-specific isotype controls, MOv18 IgG and IgE, are shown in shaded grey. Data courtesy of Dr. Panagiotis Karagiannis, KCL.

5.3.2 Cell Adhesion and Invasion Assays

Antibodies recognizing HMW-MAA have been described to have non-immunological function against cancer cells through the blockade of interactions between HMW-MAA and ECM components, resulting in the interference of metastatic processes (Chang, Campoli et al. 2004). HMW-MAA has also been associated with the modulation of cell adhesion and spreading by acting as a co-receptor for α4β1 integrin, promoting melanoma cell adhesion to fibronectin (Iida, Skubitz et al. 1992) and enhancing growth factor-regulated pathways such as FAK and ERK, which are important for tumor cell functions of cell adhesion, motility and invasion (Eisenmann, McCarthy et al. 1999; Yang, Price et al. 2004). HMW-

MAA has additionally been found to interact with matrix metalloproteinase on melanoma cells to facilitate melanoma cell invasion and promote proteolysis of ECM components such as collagen, resulting in increased tumor cell motility (Iida, Pei et al. 2001). To test if the engineered HMW-MAA antibodies of the IgG and IgE class exhibit non-immunological mechanism of action against melanoma cells *in vitro*, the ability of these engineered antibodies to restrict melanoma cell adhesion and invasion mediated by this antigen was next evaluated.

HMW-MAA has been shown to be an important contributor to melanoma adhesion and spreading to ECM components such as fibronectin (Iida, Skubitz et al. 1992) . The ability of HMW-MAA IgG and IgE antibodies to inhibit melanoma cell adhesion was evaluated *in vitro* using adhesion assays detailed in Chapter 2, Section 2.9.3. Melanoma cells coated with HMW-MAA IgG antibodies were found to significantly (P<0.01) reduce the adhesion of melanoma cells (21.7%, SD=0.6%) to fibronectin, compared to those cells incubated with the non-specific MOv18 IgG isotype control (0.4%, SD=2.2%) (Figure 5.13). A statistically significant (P<0.001) inhibition of melanoma cell adhesion was also observed for HMW-MAA antibodies of the IgE isotype (19.9%, SD=3%) compared to the MOv18 IgE non-specific control (2.2%, SD = 2.1%) (Figure 5.13). As expected, no significant difference in the inhibition of cell adhesion was seen between the HMW-MAA antibodies of IgG and IgE classes, which have identical Fab regions (Figure 5.13). Thus, engineered antibodies of the IgG1 and IgE were demonstrated *in vitro* to directly inhibit the adhesion of melanoma cells to fibronectin.

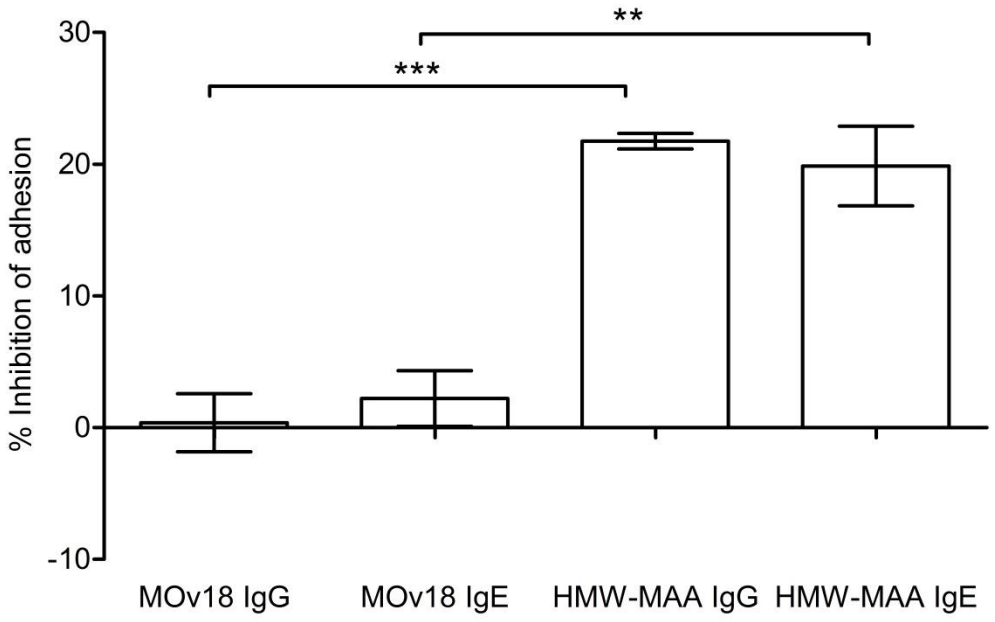


Figure 5.13 Inhibition of melanoma cell adhesion by HMW-MAA IgG and IgE antibodies. The adhesion of A-375 melanoma cells to fibronectin was evaluated following the addition of IgG and IgE antibodies targeting HMW-MAA and non-specific isotype controls, MOv18 IgG and IgE. Percent inhibition of adhesion mediated by all antibodies was calculated relative to cells treated with PBS as a 100% reference. Error bars represent one SD. The experiment was performed each time in triplicate on 3 separate days. ** $P<0.01$ and *** $P<0.001$. P values were calculated using a two-sided Students t test.

Next, the ability of HMW-MAA IgG and IgE antibodies to inhibit melanoma cell migration across a collagen layer, representing an evaluation of invasion, was analyzed using the *in vitro* assay detailed in Chapter 2, Section 2.9.4. Collagen, a basement membrane protein, represents a key barrier to the migration of tumor cells and is widely used in *in vitro* assays to evaluate for tumor cell invasion potential (Terranova, Hujanen et al. 1986). In these assays, the restriction of tumor cell migration of HMW-MAA expressing cells across a collagen layer with the addition of HMW-MAA IgG or IgE antibodies was evaluated to assess the potential of these newly engineered antibodies to restrict tumor cell invasion. A-375 melanoma cells incubated with HMW-MAA IgG antibodies were found to have a significantly reduced migratory capacity ($P<0.001$) across a collagen layer (46.7%, SD=6.9%) compared to those incubated with the non-specific MOv18 IgG isotype control (7.7%, SD=5.4%) (Figure 5.14). Similarly, cell migration was inhibited for melanoma cells incubated with the HMW-MAA IgE antibodies (46.1%, SD 7.2%) when compared to cells incubated the IgE isotype control (1.2%, SD=4.5%) (Figure 5.14). Similar to the findings of the adhesion assays examining the ability of HMW-MAA antibodies to block antigen function, no significant difference was seen in the inhibition of melanoma cell migration across a collagen layer between samples incubated with HMW-MAA antibodies of the IgG and IgE class, having identical Fab regions, in these invasion assays (Figure 5.14).

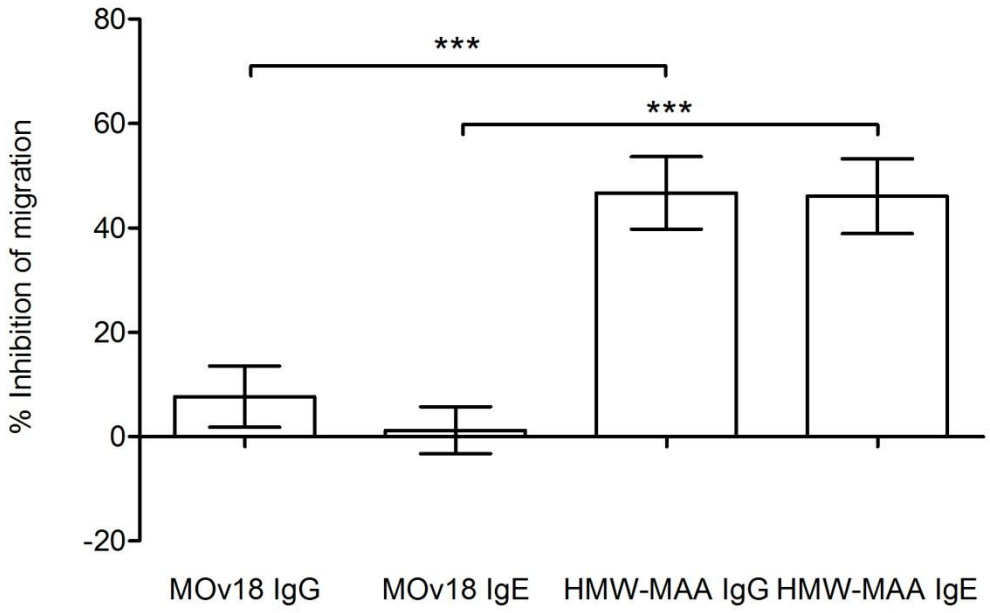


Figure 5.14 Inhibition of melanoma cell invasion by HMW-MAA IgG and IgE antibodies. The migration of A-375 melanoma cells across a collagen layer was evaluated following the addition of HMW-MAA IgG and IgE antibodies and non-specific isotype controls, MOv18 IgG and IgE. Percent inhibition of cell migration mediated by all antibodies was calculated relative to cells treated with PBS as a 100% reference. Error bars represent one SD. The experiment was performed in triplicate and repeated on separate weeks. *** $P<0.001$. *P* values were calculated using a two-sided Students *t* test.

Thus far the non-immunological functions of engineered HMW-MAA antibodies of the IgG and IgE classes have been explored *in vitro* and both antibodies demonstrated comparable impairment of melanoma cell adhesion and invasion to ECM components. The results were similar to those reported for murine versions of the antibodies evaluated in triple negative breast cancer models, which reported a 44.8% inhibition of breast cancer cell adhesion along with a 56.8% inhibition of breast cancer cell migration by co-incubation with the murine 225.28S antibody clone (Wang, Osada et al. 2010). The data presented here consequently support the ability of these engineered antibodies to inhibit the function of HMW-MAA on melanoma expressing cells.

5.3.3 Antibody-dependent Cellular Cytotoxicity and Phagocytosis Assays Using Human Primary Monocytes

While HMW-MAA antibodies have demonstrated inhibition of antigen function in relation to melanoma cell adhesion and invasion, they additionally may possess effector functions through the engagement of FcRs expressed on immune effector cells to elicit a immune cell mediated anti-tumor response. These mechanisms have to-date not been explored in the context of human immunity since only murine versions of the 225.28S antibody clone have been evaluated for Fc effector function. Therefore, engineering antibodies with human Fc regions permitted production of antibodies with the same antigen specificity but with Fc domains of different human classes, allowing for translational assessments of more clinically relevant antibodies with potentially improved effector functions.

The ability of IgG and IgE antibodies targeting HMW-MAA to engage human immune effector cells resulting in the specific killing of melanoma cells was evaluated using *in vitro* functional flow cytometry assays (described in Chapter 2, Section 2.10.4) which were designed to simultaneously measure ADCC and ADCP (Bracher, Gould et al. 2007). For these assays, primary human monocytes were freshly isolated from both patients with melanoma and healthy volunteers and used as effector cells. The use of primary immune cells allowed for the assessment of the ability of anti-melanoma antibodies to activate an individual's immune effector cells to specifically kill tumor cells. Also, by testing antibodies using both patient and healthy volunteer immune cells, any dysregulation of monocytes in disease, which many then in turn potentially limit the effector function of these antibodies, could be evaluated.

First, primary monocytes were freshly isolated from healthy volunteers (Chapter 2, Section 2.3.4), and the ability of HMW-MAA IgG antibodies to engage these cells to kill melanoma cells by ADCC/ADCP was evaluated. Melanoma cells incubated with monocytes isolated from healthy volunteers and HMW-MAA IgG were found to have a significantly ($P<0.001$) higher percentage of tumor cell death by ADCP (44.5, SD=13.4) when compared to cells incubated with MOv18 IgG isotype control (13.0, SD=4.3), or when compared to cells treated with no antibody (8.9, SD=1.5) (Figure 5.15). However, no significant increase in the percentage of ADCC was measured for cells incubated with HMW-MAA IgG antibodies (12.6, SD =2.7) compared to cells incubated with MOv18 IgG (9.4, SD=1.5), or those treated with no antibody (11.2, SD=1.7) (Figure 5.15). In summary, HMW-MAA IgG antibodies were seen to engage monocytes isolated from healthy volunteers to mediate the phagocytosis of melanoma cells in this *in vitro* assay. These results demonstrate

that HMW-MAA IgG antibodies can mediate significant Fc-mediated immune effector functions by ADCP.

Next, the ability of HMW-MAA IgG to engage FcγRs on patient monocytes to mediate ADCC and ADCP was measured. Patient monocytes were freshly isolated from three individuals with Stage II melanoma and were evaluated in these *in vitro* efficacy assays to see if cells had any restricted function. The reason for these evaluations lies in the reports of immune cell dysregulation for some immune cell populations in melanoma, such as B cells, monocytes and macrophages, which may impact on their ability to engage in tumor cell killing (Unger, Bernhard et al. 1983; Carpenter, Mick et al. 2009). No significant difference was seen in the percentage of ADCC for cells treated with HMW-MAA IgG antibodies (6.7, SD=4.8) and MOv18 IgG control antibodies (5.3, SD=4.6), or those cells treated with no antibodies (4.8, SD=4.6) (Figure 5.16). However, a significant difference (P <0.001) in the percentage of ADCP was observed between cells treated with HMW-MAA IgG antibodies (13.9, SD=2.0) and those treated with MOv18 IgG control antibodies (0.9, SD=0.2), and also for cells not incubated with any antibodies (0.9, SD=0.2) (Figure 5.16). Overall, results were similar between patient and healthy volunteer monocytes in that HMW-MAA IgG was found to significantly induce ADCP and not ADCC (Figure 5.15 & Figure 5.16).

Monocytes from both healthy volunteers and those with Stage II melanoma were both found to mediate ADCP when incubated with HMW-MAA IgG and melanoma cells. These findings suggest that this antibody may effectively activate patient effector cells against tumor cells, and therefore may harbor some potential as a therapeutic in the context of patient immunity. These results also point to the potential of HMW-MAA IgG antibodies to mediate ADCP in patients; such results

have been previously described for Trastuzumab *in vitro* when incubated with breast cancer cells and with monocytes or PBMCs (Lazar, Dang et al. 2006; Karagiannis, Singer et al. 2009). While the direct comparison of ADCP between patient and healthy volunteers may be limited by factors such as assay variability, still an increased amount of ADCP was seen from monocytes isolated from healthy volunteers. This interesting observation could potentially be explored further by performing assays using monocytes isolated from individuals with Stage III and IV melanoma which could inform of the ability of HMW-MAA antibodies to induce ADCP in patients with late stage disease.

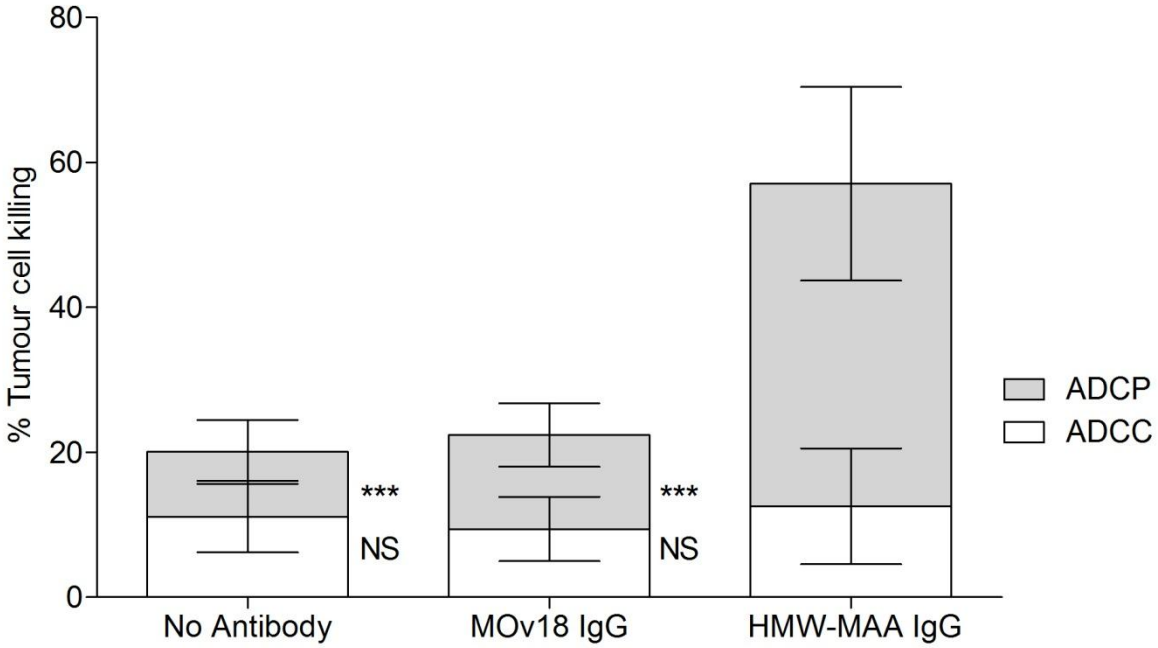


Figure 5.15 Evaluation of HMW-MAA IgG to mediate ADCC and/or ADCP against melanoma cells when incubated with primary monocytes derived from healthy volunteers. Primary monocytes isolated from healthy volunteers (n=3) were tested *in vitro* using a 3 color flow cytometric assay to simultaneously measure ADCC and ADCP of melanoma cells. Monocytes were incubated with A-375 melanoma cells and ADCC and ADCP mediated by HMW-MAA IgG were compared relative to a non-specific isotype control (MOv18 IgG) and cells treated with no antibody. Error bars represent one SD. Each condition was tested in triplicate for each of the three volunteers evaluated. NS= not significant and *** $P<0.001$. *P* values were calculated using a two-sided Student's *t* test comparing HMW-MAA IgG to either MOv18 IgG or no antibody.

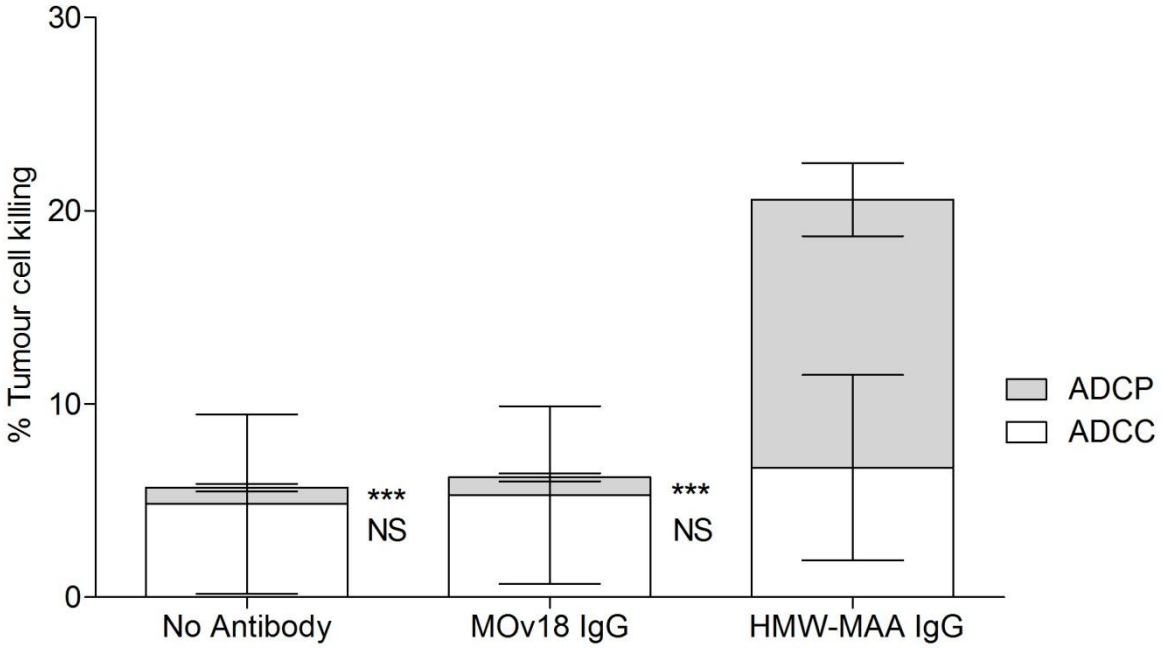


Figure 5.16 Evaluation of HMW-MAA IgG to mediate ADCC and/or ADCP against melanoma cells when incubated with primary monocytes derived from melanoma patients. Primary monocytes isolated from patients (n=3) with Stage II melanoma were tested *in vitro* using a 3 color flow cytometric assay to measure ADCC and ADCP of melanoma cells. Patient monocytes were incubated with A-375 melanoma cells and ADCC and ADCP mediated by HMW-MAA IgG were compared relative to a non-specific isotype control (MOv18 IgG) and cells treated with no antibody. Error bars represent one SD. Each condition was tested in triplicate for each of three patients evaluated. NS= not significant and *** $P<0.001$. *P* values were calculated using a two-sided Student's *t* test comparing HMW-MAA IgG to either MOv18 IgG or no antibody.

The effector functions of HMW-MAA antibodies of the IgE class were also evaluated using the same flow cytometric ADCC/ ADCP assays. Using monocytes isolated from healthy volunteers, no significant differences were observed in the percentage of ADCP between cells treated with HMW-MAA IgE (3.9, SD=2.1) and the MOv18 IgE isotype control (3.7, SD=2.2), and also between HMW-MAA IgE and the no antibody control (1.9, SD=0.8)(Figure 5.17). However, significantly higher percentages *(P* <0.01) of tumor cells were killed by ADCC when cells were incubated with HMW-MAA IgE (22.14, SD=8.6) compared to those incubated with the MOv18 IgE isotype control (7.2, SD=3.3), or those cells incubated with no antibody (5.1, SD=1.6) (Figure 5.17).

The ability of HMW-MAA IgE to engage patient monocytes to mediate the ADCC/ ADCP against melanoma cells was next evaluated in these ADCC/ADCP assays. No significant differences in the percentage of measured ADCP were seen when comparing cells incubated with the HMW-MAA IgE antibody (3.9, SD=1.6) to cells incubated with MOv18 IgE antibody (3.0, SD= 0.5), or to cells incubated with no antibody (3.8, SD=0.7) (Figure 5.18). However, a significant increase ($P < 0.001$) was measured in the percentage of ADCC for cells incubated with HMW-MAA IgE antibodies (33.1, SD=9.7) compared to those incubated with the MOv18 IgE control antibodies (10.4, SD=5.3) (Figure 5.18), or those incubated with no antibody (9.7, SD=5.0) (Figure 5.18).

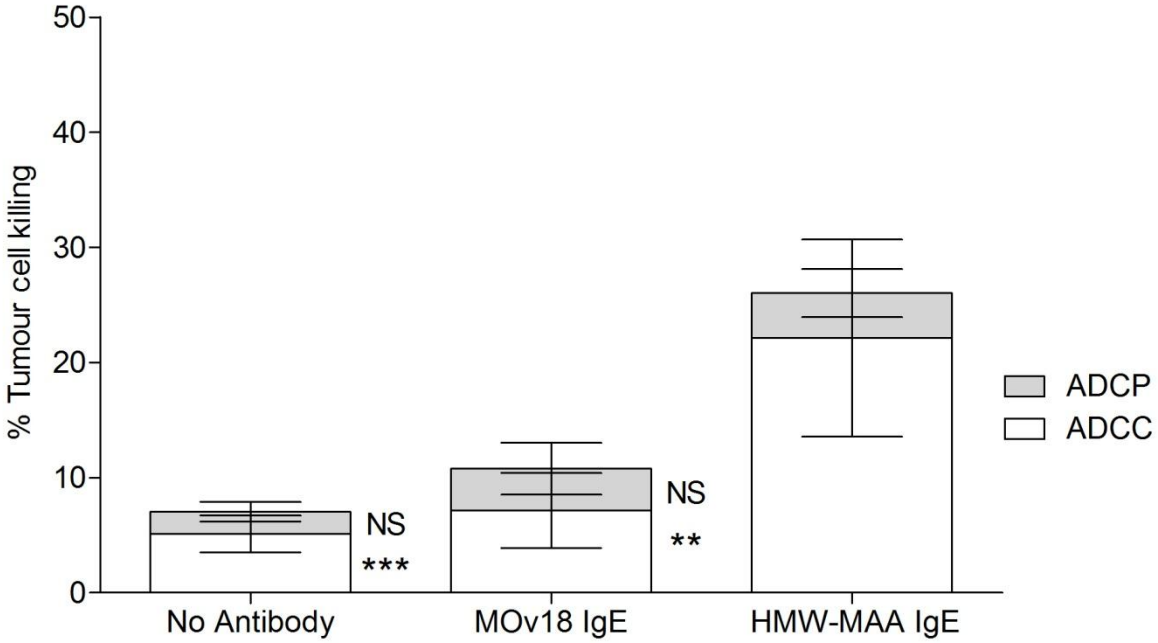


Figure 5.17 Evaluation of HMW-MAA IgE to mediate ADCC and/or ADCP against melanoma cells when incubated with primary monocytes derived from healthy volunteers. Primary monocytes isolated from 3 healthy volunteers were tested *in vitro* using a 3 color flow cytometric assay to simultaneously measure ADCC and ADCP of melanoma cells. Patient monocytes were incubated with A-375 melanoma cells and ADCC and ADCP mediated by HMW-MAA IgE were compared relative to a non-specific isotype control (MOv18 IgE) and cells treated with no antibody. Each condition was tested in triplicate for the individual evaluated. Error bars represent one SD. NS= not significant, ** $P<0.01$. *** $P<0.001$. P values were calculated using a two-sided Students t test comparing HMW-MAA IgE to either MOv18 IgE or no antibody.

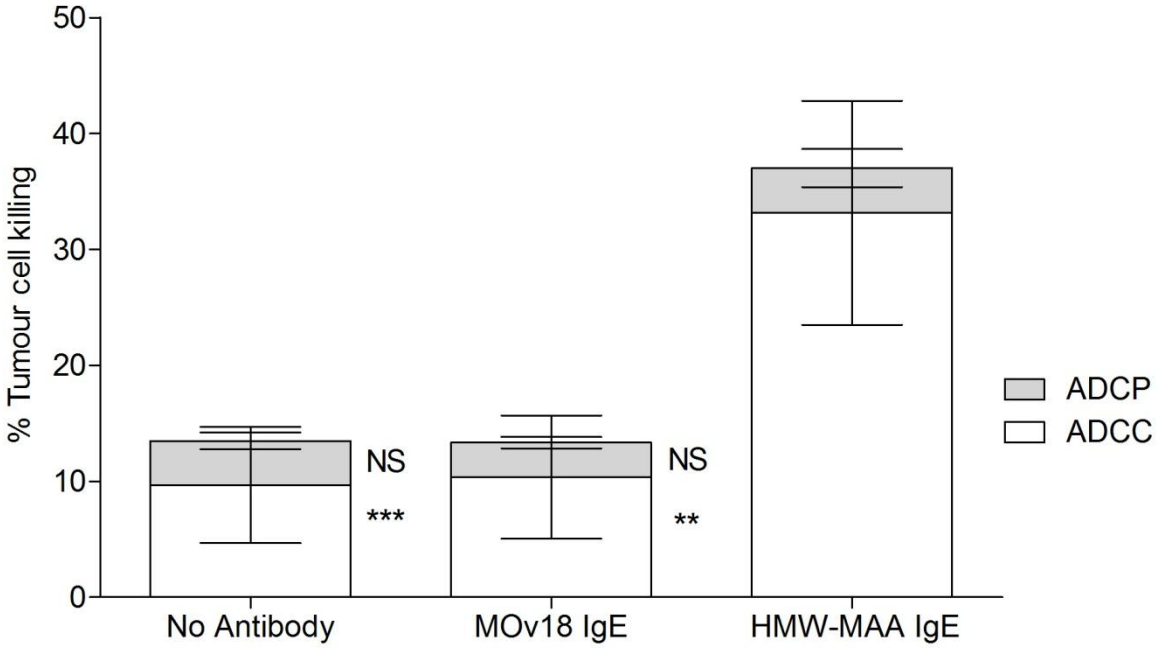


Figure 5.18 Evaluation of HMW-MAA IgE to mediate ADCC and/or ADCP against melanoma cells when incubated with primary monocytes derived from melanoma patients. Primary monocytes isolated from patients (n=3) with Stage II melanoma were tested *in vitro* using a 3 color flow cytometric assay to simultaneously measure ADCC and ADCP of melanoma cells. Patient monocytes were incubated with A-375 melanoma cells and ADCC and ADCP mediated by HMW-MAA IgE were compared relative to a non-specific isotype control (MOv18 IgE) and cells treated with no antibody. Each condition was tested in triplicate for each of the three patients evaluated. Error bars represent standard mean error. NS= not significant, ** $P<0.01$ and *** $P<0.001$. P values were calculated using a two-sided Students t test comparing HMW-MAA IgE to either MOv18 IgE or no antibody.

HMW-MAA antibodies of the IgG and IgE classes were shown to have effector functions *in vitro* through different mechanisms of action. IgG antibodies exhibited significant amounts of ADCP, while IgE mediated significant amounts of ADCC in these assays. The disparate mechanism of effector functions of these antibody classes has been described for antibodies targeting the Her2/neu antigen in breast cancer (Karagiannis, Singer et al. 2009), but these mechanisms are now shown for antibodies against melanoma herein using patient monocytes as effector cells. These results support the merit of further preclinical development of these IgG and IgE antibodies for the treatment of melanoma as single agents or potentially in combination to maximize antibody-mediated Fc effector functions in the recruitment of immune cells such as monocytes, which may elicit a more effective anti-melanoma immune response. Future studies employing these ADCC/ADCP assays could be directed at evaluating if the combination HMW-MAA IgG and IgE antibodies could enhance the effector function of a single immune cell population bearing both Fcε and Fcγ receptors, including macrophages, monocytes, and eosinophils or trigger enhanced ADCC/ADCP in mixed populations of immune cells present in solid tumors.

Monoclonal antibodies of the IgE class may hold particular potential in the treatment of melanoma due to the presence of potent immune cells in cutaneous metastases expressing FcεRs, such as mast cells and macrophages (Duncan, Richards et al. 1998; Erdag, Schaefer et al. 2012). The administration of HMW-MAA IgE antibodies in melanoma has the potential to exploit the Th2 responses previously characterized to exist in melanoma patients (Nevala, Vachon et al. 2009). Additionally, the use of an IgE monoclonal antibody targeting a tumor antigen may result in the infiltration of such immune cells normally involved in

allergic immune responses and those responses triggered in the presence of parasites. These responses have been previously observed following the treatment of tumor-antigen specific IgE antibodies in tumor-bearing animal models, and were consistent with prolonged survival and reduced tumor growth in the IgE-treated animals (Karagiannis, Wang et al. 2003). IgE cancer immunotherapy could possibly yield promising anti-tumor responses in solid tumors as suggested by *in vivo* solid tumor models (Karagiannis, Bracher et al. 2007; Teo, Utz et al. 2012), with low potential risks of systemic hypersensitivity, even in the presence of soluble forms of the targeted antigen in circulation as observed in *ex vivo* safety models evaluating a tumor-specific IgE antibody (Rudman, Josephs et al. 2011).

5.4 Conclusions

This chapter described the discovery, characterization and *in vitro* functions of novel anti-melanoma monoclonal antibodies. First, the antibody repertoire of melanoma patients was harnessed to discover novel tumor-specific antibodies. One such patient-derived melanoma-specific monoclonal antibody, M111_2G3, mediated ADCC of tumor cells *in vitro*, suggesting the merit of this approach to discover anti-melanoma antibodies with therapeutic potential. While the amounts of available M111_2G3 antibody were limited, further *in vitro* functional assays were employed using in-house engineered monoclonal antibodies recognizing a melanoma associated antigen. These *in vitro* assays could be applied in future

evaluations of the possible mechanisms of action of patient-derived tumor specific antibodies to specifically kill or restrict the adhesion or invasion of tumor cells.

Engineered monoclonal antibodies of the IgG and IgE classes were found *in vitro* to partially restrict tumor cell adhesion and invasion. Furthermore, these IgE and IgG antibodies were capable of activating patient peripheral blood monocytes to kill melanoma cells *in vitro* by disparate mechanisms, one by ADCC and the other by ADCP, respectively. Developing therapeutic anti-melanoma antibodies of the IgE class may offer a novel strategy to increase ADCC against melanoma cells.

Patient-derived melanoma-specific antibodies and those targeting the HMW-MAA bearing human Fc regions all collectively offer new immunotherapeutic approaches for the treatment of melanoma and warrant further preclinical assessments in disease-relevant *in vitro* and *in vivo* models. Such *in vitro* models would permit the examination of the HMW-MAA antibody's potential to inhibit cell proliferation and colony formation along with its ability to engage human patient-derived effector cells to perform Fc-mediated functions such as CDC and ADCC/ADCP, including the assessment of additional immune cell types as effector cells. Analyses of these antibodies with human Fc regions *in vivo* are also essential for future preclinical evaluations of efficacy and should include the use of disease-relevant animal models of melanoma such as those described by Chu and colleagues, which use human tumors transplanted into mice engrafted with human immune effector cells (Chu, Ali et al. 2012).

Chapter 6: Conclusions and Future Work

6.1 Introduction

The recent clinical success of the monoclonal antibody Ipilimumab has generated renewed interest in the use of antibody therapeutics in melanoma. Not only does Ipilimumab signify the approval of the first monoclonal antibody for the treatment of melanoma but, notably, it is the first antibody therapeutic that functions by modulating regulatory mechanisms to overcome tolerance and heighten the host adaptive immune response against cancer. These attributes highlight the importance of antibodies as a class of drugs to treat melanoma and the merits of dissecting immunomodulatory pathways in cancer with the aim of harnessing host anti-tumor immune responses. Key to the success of Ipilimumab were the understanding of the nature of T cell responses to melanoma and the devising of a drug that would lead to the activation and proliferation of T cells to enhance the host anti-tumor cellular immune response. Further understanding of the nature of other immune cell populations and their response to cancer could pave the way for additional therapeutic approaches to treat melanoma and other cancers.

The purpose of this thesis was to investigate the humoral immune response to melanoma from a patient cohort and explore the possible anti-tumor functions of patient-derived and engineered antibodies against melanoma cell surface antigens. The findings presented in this thesis highlight the prevalent humoral immune response to melanoma across a 21 patient cohort, provide novel insights into the modulation of antibody subclasses in the cutaneous metastatic melanoma microenvironment, and describe approaches to discover and characterize novel anti-melanoma antibodies, all of which are highly applicable to future immunotherapeutic strategies for the treatment of melanoma.

6.2 Humoral Immune Response to Melanoma

Two key contributions of this thesis provide further understanding of the humoral immune responses to melanoma. Firstly, the *ex vivo* culture of patient and healthy volunteer B cells along with the development of the cell-based ELISA (Chapter 3, Section 3.3.3) allowed for the broad analysis of host antibody responses to antigens displayed on the surface of melanoma cells (Chapter 4, Section 4.1). Secondly, the study of IgG subclass composition resulted in the observation of increased proportional production of antibodies of the IgG4 subclass in the cutaneous metastatic melanoma microenvironment compared to the periphery (Chapter 4, Section 4.2), which has not been previously described. Each of these findings contributes to the further understanding of humoral immune response to melanoma and provides a fresh perspective on an arm of the adaptive immune response which has received little focus until now.

6.2.1 Monitoring the Melanoma-reactive IgG Compartment from Patient Memory B cells

The development of a cell-based ELISA herein has allowed for a broad analysis of the humoral immune response to antigens displayed on the surface of melanoma cells. This approach has expanded the scope of characterization of the humoral immune response to cancer in several ways. Firstly, the use of whole cells allowed for the assessment of antibody reactivity to a multitude of antigens residing on the cell surface. These are of particular interest in the study of humoral immunity against melanoma because these antibodies recognize cell surface antigens and may harbor the potential to mediate CDC, ADCC and ADCP against tumor cells

upon recognition of their targeted antigen. Secondly, this work presents an assay that could potentially be used as a standardized technique to study anti-tumor antibody responses in melanoma and other cancers, something that is currently lacking (Reuschenbach, von Knebel Doeberitz et al. 2009). This readout is not limited by the availability of commercially available recombinant antigens or their recombinant production because it makes use of readily available cells. Thirdly, assessments of total immunological memory can be made with this approach through the examination of antibodies produced from memory B cells rather than the evaluation of antibodies resident in the sera at the time point of sampling (Lanzavecchia, Bernasconi et al. 2006). Prior to this work, serological studies have provided valuable insight into humoral immune responses to melanoma by examining antibody responses to autologous cells (Lewis, Ikonopisov et al. 1969) along with antibody responses to several selected recombinant antigens (Stockert, Jäger et al. 1998; Trefzer, Hofmann et al. 2006). However, these more extensive serological studies examined melanoma antigens that are predominantly cytoplasmic or expressed in the nucleus, and the selection of such antigens has been variable across studies. The approach described herein has general applicability and could be used to identify the presence of a memory B cell compartment containing tumor-reactive antibodies against cell surface antigens of any adherent cell type in different cancers and patient groups. The approach could even be employed to monitor humoral immune responses prior to and following therapeutic treatments, such as vaccines aimed at enhancing humoral immunity, or therapeutic antibodies aimed at overcoming immune tolerance, or even following the administration of small molecule drugs, such as BRAF kinase inhibitors, to evaluate possible effects on tumor-specific antibody responses in a similar manner to those studies evaluating T cell function and tumor-specific T cell

responses following administration (Boni, Cogdill et al. 2010; Hong, Vence et al. 2012). Thus, the development of a cell-based ELISA to measure antibody reactivity from *ex vivo* B cell cultures not only complemented previous serological studies but has also allowed for the more general evaluation of the reactivity of antibodies to melanoma cells expressing a multitude of antigens along with the assessment of the immunologic history of the patient humoral immune response to antigens residing on the surface of melanoma cells.

A melanoma-reactive antibody compartment was found when examining patient memory B cells among a cohort of patients diagnosed with Stage I, II, III and IV melanoma (Chapter 4). The specificity of the host antibody response to melanoma was supported by the finding that antibodies derived from a cohort of healthy volunteers had significantly lower responses to melanoma cells compared to the patient cohort (Figure 4.2 & Figure 4.3). Such a broad humoral immune response to melanoma has not been previously described among patients with non-metastatic and metastatic disease. This could be because past serological evaluations have focused on selected antigens and only provided insight into antibody responses against these specific antigens (Stockert, Jäger et al. 1998; Trefzer, Hofmann et al. 2006), many of which are lost or acquired during disease progression (Barrow, Browning et al. 2006). The use of whole melanoma cells in the cell-based ELISA allowed for the evaluation of antibody reactivity against a wide spectrum of antigens, possibly some as of yet unidentified, lending a broader characterization of humoral immune responses. Additionally, the use of non-autologous cells in the cell-based ELISA demonstrated that these melanoma-reactive antibodies were not just specific to autologous cancer cells, but rather the antibodies recognize cell surface antigens common to other individuals, which

could be a particularly meaningful approach to discovering novel antibodies from patients to be used as a passive immunotherapy. This work identified the presence of a circulating memory B cell compartment in the blood of most patients, capable of producing melanoma-reactive antibodies, thus illustrating the overall widespread immunologic B cell memory to melanoma among patients with varying stages of disease.

Monitoring the circulating memory B cell compartment from a 21 patient cohort revealed that antibody reactivity to melanoma cells was decreased in relation to disease progression (Chapter 4, Section 4.2.3). In this thesis, it was first evaluated whether the decrease in antibody responses in patients with progressing disease was solely a function of a decrease in circulating CD27+ memory B cell populations. Interestingly, no significant difference was observed between the proportion of the peripheral blood CD27+ memory B cell compartments in the blood of non-metastatic compared to that of metastatic melanoma patients (Figure 4.1), suggesting that the decrease in melanoma-reactive antibodies was not entirely a function of the reduced peripheral blood memory B cell compartment with progressing malignancy. It could be hypothesized, following the immunoediting paradigm (Dunn, Bruce et al. 2002; Schreiber, Old et al. 2011), that such findings may in part be a result of changes in the phenotype of the tumor. These may arise from the selective pressure of the immune system on cancer cells, allowing the survival of non-immunogenic cancer cells with decreased antigenicity and with progressing malignancy. A decrease in the clonal expansion of these melanoma-reactive B cells may follow in the absence of antigen. Thus, the reduction of melanoma-reactive antibodies with disease progression may represent weakened host humoral immune responses due to the decreased

antigenicity of tumor cells with progressing disease. This is supported by studies demonstrating the loss of some melanoma antigens, such as differentiation antigens, with progressing disease (Trefzer, Hofmann et al. 2006), along with serological studies examining antibody reactivity to autologous cells, which were shown to decrease in patients with advanced malignancy (Lewis, Ikonopisov et al. 1969). Other mechanisms may also be at play such as the exhaustion of antigen-reactive B cells, or immunomodulatory responses triggering less potent humoral responses. In summary, the findings herein have expanded upon the characterization of memory B cell homeostasis in melanoma by demonstrating a decreased reactivity of antibodies produced from *ex vivo* activated patient memory B cells to melanoma cells. The use of allogeneic melanoma cells in these evaluations, which display a multitude of cell surface antigens that are likely commonly expressed across individuals, adds a more general evaluation of the decreasing reactivity of antibodies to a larger set of antigens than those previously described for autologous cells or even single antigens.

Further examination of the memory B cell compartment in patients was performed to estimate the frequency of melanoma-reactive antibody responses in patients. The frequency of B cells producing melanoma-reactive antibodies to multiple metastatic melanoma cell lines, with each cell line derived from a different individual, was estimated to be similar to B cell cultures derived from one individual (Figure 4.7). These findings suggest the possibility that these metastatic cell lines share similar or common cell surface antigens. Additionally, B cells tested from different individuals diagnosed with Stage II melanoma were estimated to have comparable frequencies of B cells producing melanoma-reactive antibodies, approximately 1 in 2000 B cells (Figure 4.8). These estimations

revealed the low to moderate frequencies of B cells capable of producing melanoma-reactive antibodies residing in patients diagnosed with Stage II disease, compared to the much higher frequencies of antigen-specific B cells present following vaccination for measles virus, tetanus toxin and varicella zoster virus antigens, which were reported for one individual to be 1 in 50, 1 in 400, and 1 in 585 B cells, respectively (Pinna, Corti et al. 2009). While the frequency of B cells producing antibodies to TAAs has not been previously described in patients, the frequency of T cells reactive to TAAs such as tyrosinase has been reported to be as high as 2.2% of total CD8+ T cells in one of six individuals studied with metastatic disease, which roughly translates to 1 in 50 CD8+ T cells (although this finding was variable among individuals since 4 patients did not have tyrosinase reactive CD8+ T cells and one patient had reactivity of 0.19%, roughly 1 in 500 CD8+ T cells) (Lee, Yee et al. 1999). While it has been demonstrated that patient memory B cells capable of producing anti-tumor humoral immune responses exist in the periphery in somewhat low to moderate frequencies, it is not known whether these B cells are capable of mounting significant anti-tumor responses in patients, although strategies to enhance these host responses could be of interest as a potential therapeutic approach.

The presence of B cells producing melanoma-reactive antibodies across patients was demonstrated in this thesis and the activation of this arm of adaptive immunity in patients could be of therapeutic value. This claim is also supported by a recent study analyzing immune cell infiltration into metastatic melanoma tumors and patient survival, which found that the group of patients (8%) with diffuse immune cell infiltrates in the tumor contained B cells (25%) and plasma cells (8%) among other immune cells. It was found that the presence of these B linage cells

correlated with increased rates of survival of 130 months compared to groups with no immune cell infiltrates or perivascular infiltrates, who had survival rates of 15 and 23 months, respectively (Erdag, Schaefer et al. 2012). Taken together, these findings hint at the possible anti-tumor role of B cells and lend support for the exploration of therapeutic attempts to enhance the frequency of specific sets of B cells and perhaps trigger the production of anti-melanoma antibodies. It could be speculated that such attempts may have already been made to enhance host humoral immunity to melanoma, although the role of B cells in these attempts has not been fully dissected. For example, in adoptive cell therapies *ex vivo* activation of TILs through the use of IL-2 is primarily thought to expand anti-tumor responses mediated by CD8+ T cells, but these mixed lymphocyte populations also contain B cells, which can also proliferate in the presence of IL-2 (Mingari, Gerosa et al. 1984; Dudley, Yang et al. 2008; Besser, Shapira-Frommer et al. 2010). Therefore, it is conceivable that antibodies from these TILs also contribute to the anti-tumor response. It is possible that further targeted activation of specific B cell subsets by a TLR9 agonist could enhance B cell mediated anti-tumor responses.

In the 21 patients studied, no clear relationship between the antibody reactivity of *ex vivo* activated B cells to melanoma cells and clinical outcome was found, though the follow-up was limited to between 6 and 24 months. Longer follow-up of a larger patient cohort may help identify any correlations between melanoma-reactive mature memory B cell responses and disease progression. In this study, of note was one Stage IV patient with the highest % of tumor-reactive cultures (92%, Patient 72) in the patient cohort, who is a long-term survivor (8 years) with stable disease. However, in order to draw any meaningful conclusions from this observation it would be important to observe similar findings in other long-term

survivors with metastatic disease. It could be speculated that host immunity has profound anti-tumor effects in some patients and could be the mechanism behind some reports of "spontaneous" remissions or long term stable disease; indeed, one such case reported the infiltration of plasma cells and lymphocytes into the areas of tumor necrosis prior to long term remission (Bulkley, Cohen et al. 1975). While correlations between lymphocyte infiltration in primary melanomas and improved prognosis have been made (Clark, Elder et al. 1989; Clemente, Mihm et al. 1996) along with B and plasma cell infiltration in metastatic tumors (Erdag, Schaefer et al. 2012), the effects of these cells and their production of anti-melanoma antibodies in sufficient quantities to mount a meaningful anti-tumor response in patients still remains unclear. These published observations together with data provided in this thesis highlight the importance of further dissecting the nature, tumor specificity and potency of humoral responses in patient circulation as well as in the tumor microenvironment, and the critical need to assess how the presence of different B cell subsets impact on the clinical course of melanoma.

The widespread presence of memory B cells producing melanoma-reactive antibodies in this patient cohort, which included patients with advanced disease, suggests that humoral immune responses of sufficient potency may not be present to mount significant anti-tumor responses, and that these responses may be weakened with progressing disease. There is also some evidence that the activation or functions of such memory B cells and the antibodies they produce may be impaired in melanoma, as seen by the hyporesponsiveness of metastatic melanoma patient memory B cells to stimuli such as CD40 and TLR9 (Carpenter, Mick et al. 2009). Immunoregulatory elements in the tumor microenvironment may also modulate and reduce the potency of any melanoma-reactive antibodies

produced. These may include the expression of Fc-decoy receptors on melanoma cells (Cassard, Cohen-Solal et al. 2008), the impairment of cell functions of immune cell populations, such as NK or macrophages, rendering them incapable of mediating cytotoxic killing such as ADCC/ADCP in solid tumors (Gordon and Freedman 2006; Pietra, Manzini et al. 2012), or even the possible suppression of antibody production by modulatory factors in the tumor microenvironment. Understanding and altering these modulatory mechanisms may hold promise for the design of novel immunotherapies for cancer.

6.3 IgG4 in the Cutaneous Metastatic Tumor Microenvironment

The finding of a polarization of IgG subclass production towards IgG4 in the metastatic melanoma cutaneous tumor microenvironment represents a novel discovery of this thesis. The role of IgG4 in disease pathogenesis is newly emerging with the identification of IgG4-related inflammatory diseases such as autoimmune pancreatitis (Stone, Zen et al. 2012). In cancer, altered levels of IgG4 in the sera of some metastatic melanoma patients have been previously described (Daveau, Pavie-Fischer et al. 1977), along with the recent evidence of the infiltration of IgG4+ plasma cells in cholangiocarcinomas, malignancies of the biliary duct system (Harada, Shimoda et al. 2012). Beyond these two reports, the relationship between IgG4 and cancer has largely been unexplored.

Following the observation of IgG4 polarization in the tumor, the influence of Th2 cytokines on humoral immunity were explored. Alterations of humoral immune

responses, such as antibody class and subclass proportions by Th2 cytokines, have been previously described including the effects of IL-4 on IgE and IgG4 class switching along with the enhancement of IgG4 production by IL-10 (Jeannin, Lecoanet et al. 1998; Satoguina, Weyand et al. 2005). Increased levels of Th2 cytokines have been described in melanoma and other cancers with increases in the levels of Th2 cytokines, such as IL-4 and IL-10, in patients (Sheu, Lin et al. 2001; Agarwal, Verma et al. 2006; Nevala, Vachon et al. 2009). To explore if Th2 cytokines in the tumor microenvironment can alter IgG subclass production, experiments were performed in which metastatic melanoma patient B cells, autologous PBMCs, and A-375 metastatic melanoma cells were co-cultured *ex vivo*. Results from these co-culture experiments revealed that increased levels of IgG4 production were only observed in culture conditions which contained both A-375 melanoma cells and PBMCs that were measured to have increased levels of IL-4 and IL-10, and not in culture conditions with B cells cultured with only PBMCs or A-375 cells (Figure 4.12). Interestingly, the skewing of antibody subclasses was not seen in these co-culture assays in the absence of melanoma cells, which have been shown to secrete IL-10, including the A-375 cell line (Chen, Daniel et al. 1994; Sumimoto, Imabayashi et al. 2006), suggesting these cells could be an important source of this regulatory cytokine, resulting in the favored production of IgG4 in the tumor microenvironment.

The findings of an increased proportional production of antibodies of the IgG4 subclass and relative decrease in the proportion of the IgG1 subclass may represent a mechanism by which humoral immunity is impaired in melanoma. This would be because of the reduction in the production of IgG1 antibodies in the tumor, the antibody class thought to have the most potent immune effector

functions, accompanied by the increase in IgG4, which is generally thought to have the least potent Fc effector functions among the four IgG subclasses (van der Zee, van Swieten et al. 1986; Bruggemann, Williams et al. 1987; Aalberse, Stapel et al. 2009). If such ratios were to apply to melanoma-reactive antibodies, the reduced capacity of tumor-specific antibodies to mediate CDC, ADCC and ADCP could result in a weakened anti-tumor antibody response in the metastatic cutaneous tumor microenvironment. The changes in antibody subclass proportions suggest another mechanism of immunomodulation in melanoma and possibly other cancers. Further studies are required to dissect these immunomodulatory mechanisms affecting antibody functions in cancer. Strategies aimed at "re-polarizing" the tumor microenvironment towards IgG1 production, such as blocking IL-10, could possibly have merit in increasing the potency of any tumor-specific humoral immune responses present in the tumor.

6.4 Characterization and Functional Studies of Antibodies Targeting Melanoma Antigens

The work presented in this thesis concentrated on two novel approaches that warrant further development and consideration as tools to discover future therapeutics for melanoma and other solid tumors. The first approach is the potential use of patient-derived melanoma-reactive IgG antibodies for passive immunotherapy. The second approach focuses on comparing the efficacy of chimeric antibodies of the IgG1 and IgE classes with human Fc regions and variable regions recognizing the characterized melanoma antigen HMW-MAA as potential therapeutic agents.

6.4.1 Engineered Antibodies of the IgE and IgG1 Classes Targeting HMW-MAA

The HMW-MAA represents an ideal target for a monoclonal antibody therapeutic because of its restricted distribution on normal tissue and high expression on melanoma and other cancer cells (Campoli, Chang et al. 2004). Blockade of this antigen with monoclonal antibodies has revealed several non-immunological roles of HMW-MAA (Chapter 2, Section 1.2.3.1) which may aide the enhancement of the metastatic potential of cells expressing this antigen (Price, Colvin Wanshura et al. 2011). Antibodies recognizing HMW-MAA, such as the 225.28S murine antibody clone, have been shown to restrict tumor growth in murine models of cancer (Hafner, Breiteneder et al. 2005). The work herein has evaluated a chimeric version of the 225.28S HMW-MAA antibody engineered in-house and its direct

Fab-mediated functions to block melanoma cell adhesion and migration, along with its human Fc-mediated effector function to recruit innate immune effector cells resulting in the targeted destruction of melanoma cells. In addition to an IgG1 antibody, an engineered chimeric antibody with human Fc regions of the IgE class targeting the HMW-MAA was also evaluated. Naturally occurring IgE responses are normally targeted against allergens but also protect against parasitic infections and perform effector functions against pathogens such as ADCC and ADCP. It has been proposed that therapeutic antibodies of the IgE class hold promise for the treatment of solid tumors, such as breast and ovarian cancers, by re-directing high-affinity IgE receptor-expressing effector cells against tumor cells to harness allergic reactions in tumors (Karagiannis, Josephs et al. 2012). To-date the concept of IgE immunotherapy for solid tumors has not been tested in melanoma. Melanoma may serve as an ideal tumor type for IgE immunotherapy due to the high infiltration of FcεR bearing immune cells in tumors such as mast cells and macrophages (Duncan, Richards et al. 1998; Tth, Tth-Jakatics et al. 2000; Erdag, Schaefer et al. 2012).

Functional evaluations of HMW-MAA IgG and IgE antibodies with human Fc regions were conducted as a part of this work to assess the non-immunological functions of these engineered antibodies to restrict tumor cell adhesion and migratory capacity (Chapter 5, Section 5.3.2). The 225.28S antibody clone binds to an epitope of the core glycoprotein of HMW-MAA, a region that has been described to contain integrin and collagen binding sites (Wilson, Imai et al. 1981; Kantor, Albino et al. 1986; Price, Colvin Wanshura et al. 2011). The *in vitro* experiments performed herein demonstrate that both the 225.28S IgG and IgE antibodies partially block the interactions between melanoma cells and ECM components

such as fibronectin and collagen, thus restricting cell adhesion and migration *in vitro* (Figure 5.13 & Figure 5.14). These experiments using newly engineered chimeric 225.28S antibodies were similar to those performed by Wang and colleagues using the original murine 225.28S antibody clone in triple negative breast cancer models (Wang, Osada et al. 2010).

Furthermore, the immunological function of HMW-MAA IgG and IgE antibodies was examined through the use of engineered antibodies with human Fc regions and effector cells isolated from both healthy volunteers and patients. These Fc-FcR mediated functions of the 225.28 clone have not been previously examined, since the original clone bears murine Fc regions which are not expected to effectively activate human immune effector cells to provide meaningful evaluations of effector function in humans (Bergman, Basse et al. 2000). These *in vitro* studies revealed that HMW-MAA IgG and IgE antibodies mediated ADCP or ADCC against tumor cells through the engagement of FcRs on human monocytes, which are known to express FcγRs and FcεRs (Maurer, Fiebiger et al. 1994; Karagiannis, Wang et al. 2003). These IgG1 and IgE antibodies were observed to mediate disparate mechanisms of action, ADCP and ADCC, respectively. These findings are in concordance with equivalent data reported in relation to effector functions of IgG and IgE antibodies recognizing the Her2/neu antigen expressed on breast cancer cells (Karagiannis, Singer et al. 2009). Along with the engineered anti-Her2/neu IgE homologue of Trastuzumab, another tumor specific antibody of the IgE class, MOv18 IgE, has demonstrated Fcε-mediated ADCC using *in vitro* and *in vivo* models of ovarian cancer. This agent is currently undergoing preclinical development leading to a Phase I study (Karagiannis, Wang et al. 2003; Karagiannis, Josephs et al. 2012). In addition to the well-described ability of tumor-specific antibodies of

the IgG class to mediate ADCC (Reichert 2012; Scott, Wolchok et al. 2012), tumor-specific antibodies of the IgE class may hold promise for the treatment of melanoma and other solid tumors through the engagement of FcεR bearing immune effector cells present in the tumor, such as monocytes/macrophages resulting in ADCC and ADCP. These findings highlight the potential of antibodies of the IgG and IgE class targeting HMW-MAA to engage cells of the innate immune system in patients to mount an anti-tumor response, albeit through different mechanisms of action.

Both the non-immunological and immunological studies presented in this thesis provide early evaluations of these chimeric HMW-MAA antibodies and are expected to further support the preclinical development of IgG antibodies bearing human Fc regions targeting HMW-MAA along with the development of antibodies engineered with Fc regions of the IgE class.

6.4.2 Patient-derived Antibodies

There has been renewed interest in the study of human antibody repertoires as a potential source of novel antibodies, particularly in light of the more recent reports describing techniques to activate human B cells in *ex vivo* cell cultures (Traggiai, Becker et al. 2004; Kwakkenbos, Diehl et al. 2010). The focus of such an approach has primarily been in the field of infectious diseases with the aim of discovering neutralizing antibodies to viruses. The study of antibody responses in infectious diseases provides an ideal model system, considering the availability of virus antigens for use in the screening for reactive antibodies and the feasibility of studying antibody responses following routine vaccinations. More challenging is

the application of this approach to cancers such as melanoma, even with described immunogenicity and documented humoral immune responses. The reasons include disease heterogeneity, particularly with regard to antigen expression (Stockert, Jäger et al. 1998; Barrow, Browning et al. 2006; Trefzer, Hofmann et al. 2006); possible effects of disease on B cell homeostasis, such as collapse of memory B cell compartments in metastatic patients (Carpenter, Mick et al. 2009); immunomodulatory mechanisms in cancer potentially altering the effectiveness of humoral immune responses; and lack of commercially available cell surface antigens.

Through the application of more recent methods for the *ex vivo* culturing of human B cells (Traggiai, Becker et al. 2004), this thesis widely examined humoral memory to melanoma in a patient cohort by devising a medium-throughput screening tool using cancer cells (Chapter 3). This approach led to the isolation of monoclonal antibodies from patients with melanoma, some of which were characterized to have specificity to non-autologous melanoma cell lines but not to normal cells such as melanocytes. These findings support the approach outlined in this thesis (Chapter 5, Section 5.2.1) to discover, characterize, and evaluate potential cytotoxicity of anti-melanoma antibodies from patient memory B cells. One such melanoma-reactive antibody clone, M111_2G3, was found to mediate cytotoxicity in the presence of melanoma cells and human monocytic effector cells (Chapter 5, Section 5.2.2), highlighting the cytotoxic potential of such antibodies and their possible role in engaging innate immune cells to mount an anti-tumor response. The identification of melanoma-reactive antibodies, particularly those that appear to specifically bind to melanoma cells such as the M111_2G3, supports the merit of this approach to discover monoclonal antibodies from patients with cancer.

Furthermore, the finding described herein that memory B cells producing melanoma-reactive antibodies were observed across many individuals in the cohort studied and not restricted to particular clinical scenarios, or to just a few select individuals, or those undergoing specific immunological or other treatments, further supports the value of this approach to discovering novel antibodies by harnessing the B cell memory responses of patients with cancer.

The antibody discovery methodology described herein could also be applied to identifying antibodies from other immunogenic cancers with reported humoral immune responses against tumor associated antigens such as breast, lung, and ovarian cancers (Lu, Goodell et al. 2008; Reuschenbach, von Knebel Doeberitz et al. 2009). This approach also may lead to the identification of novel tumor-associated antigens in melanoma and in other cancers, and the study of such antigens could then in turn reveal novel pathways associated with these antigens which contribute to malignancy.

6.5 Future Work

6.5.1 Discovery and Preclinical Development of Patient-derived Antibodies for Immunotherapy

Patient-derived melanoma-reactive antibodies were observed to have the capacity to mediate cytotoxic effects against melanoma cells *in vitro*, and these findings support the merit of the approach presented in this thesis to discovering novel antibodies. While this methodology resulted in the production of antibodies in sufficient amounts for initial characterization and preliminary functional assays, the combination of this approach with the engineering of recombinant antibodies would be needed to permit the greater characterization of melanoma-reactive antibodies. Further characterization of the antibody clone identified in this thesis and others discovered using this approach would include antigen identification by mass spectrometry, antibody-antigen affinity measurements, evaluation of antibody specificity to tumors using an array of melanoma lesions from different individuals, and evaluations of reactivity to other tumors types using tissue arrays, as well as reactivity to normal tissues. Additionally, and depending upon the functions of the particular antigenic target on tumor cells, a more comprehensive study of possible non-immunological functions should be conducted, including studies evaluating the blocking of receptor-ligand interactions and the hindering of downstream signaling events that could block tumor cell activation. Other evaluations may include the direct inhibition of cell proliferation, colony formation, adhesion and invasion, among others. In the future, selected antibodies could be evaluated for their potential efficacy as passive immunotherapeutic agents by using animal models of different cancers that express the particular antigens. In the context of melanoma, animal models of melanoma, such as those

described by Chu and colleagues, should be engrafted with human effector cells so that direct and Fc-FcR mediated functions of antibodies can be evaluated in relation to efficacy (Chu, Ali et al. 2012). All of these evaluations would collectively serve as steps to evaluate the potential of novel antibodies derived from patients as possible immunotherapeutic agents.

6.5.2 Role of IgG4 in Cancer

The work presented in this thesis constitutes the third report of alterations in IgG4 production or IgG4+ plasma cell infiltration in cancer, and the first report to have described altered production of IgG4 in the cutaneous melanoma microenvironment. While these reports are intriguing findings, the association between IgG4 and cancer is not clear and warrants further exploration. A first step would be to evaluate if there is an association between IgG4 and multiple cancer types, which could be achieved by analyzing tissues from different tumor types for IgG4+ B cell infiltration by immunohistochemistry. Additionally, similar studies to those performed in this thesis could be conducted to evaluate the specificity of the different IgG subclasses to tumor cells expressed by B cells residing in excised tumors, including the IgG4 antibodies. Furthermore, it is important to further elucidate the exact mechanisms that trigger IgG4 polarization in the tumor microenvironment and whether these responses could be re-directed using novel therapeutic approaches.

The possible biological significance of IgG4 in cancer has been largely unexplored. The alteration of the proportion of IgG subclasses resulting in increased IgG4 and decreased IgG1 proportional production in cutaneous metastatic melanoma

tumors as described in this thesis could represent an impairment of humoral immunity in the tumor microenvironment. This hypothesis has been tested by colleagues by engineering antibodies of the IgG1 and IgG4 subclasses against a melanoma cell surface antigen (HMW-MAA) to compare the ability of these subclasses to engage immune effector cells to mediate ADCC and ADCP using *in vitro* flow cytometric assays (Bracher, Gould et al. 2007), and also in animal models of melanoma that are engrafted with human immune effector cells (Chu, Ali et al. 2012). These experiments have illustrated the weak Fc effector function of IgG4 compared to IgG1 to mediate ADCC/ADCP *in vitro* and *in vivo*, along with the reduction in the efficacy of IgG1 antibodies in the presence of IgG4 (Karagiannis, Gilbert et al. manuscript under review). Further studies are currently underway evaluating the mechanisms by which IgG4 antibodies impair humoral immunity, and these will shed fresh perspective on the roles of redirected humoral responses in melanoma.

6.5.3 Preclinical Development of HMW-MAA IgE and IgG Antibodies

To further evaluate the potential clinical application of HMW-MAA IgG and IgE antibodies, additional assessments of the efficacy of these antibodies bearing Fc regions are required. While these antibodies were observed to engage human immune cells *in vitro* to mediate ADCC and ADCP, such evaluations should also be carried out using *in vivo* models of melanoma. Using human immune effector cells, the Fc-mediated contribution of these antibodies to restrict tumor growth could be more fully ascertained in animal models of melanoma, and such work is presently being carried out by colleagues. It would also be of interest to evaluate the efficacy

of IgG and IgE classes administered in combination, to test if these antibody classes could simultaneously engage different immune cells or even act in concert to increase the activation of immune cells bearing both FcεRs and FcγRs resulting in an increased restriction of tumor growth compared to the administration of individual antibody agents.

Thus far therapeutic antibodies of the IgE class have yet to be administered in humans and the preclinical development of an IgE antibody targeting HMW-MAA would require additional safety studies. It is proposed that tumor-specific antibodies of the IgE class may exploit potent immune responses, normally known to operate in allergic inflammation and in immune protection against parasitic infections, within the tumor, such as the activation of FcεRs on tumor-resident immune cells leading to ADCC and ADCP of tumor cells (Karagiannis, Josephs et al. 2012). However, if the antigen is shed in the circulation at high enough levels and in a multivalent form, the occurrence of such mechanisms in the blood could trigger type 1 hypersensitivity, which could lead to anaphylaxis. Although the exact requirements for an antigen to trigger anaphylaxis is not fully understood, it is hypothesized that such crosslinking of therapeutic IgE antibodies on tumor antigens and FcεR expressing immune cells would be rare events in patient blood, but should nevertheless be examined for each antibody candidate in the context of individual therapeutic indications. This concept has so far been examined as part of the preclinical development of the anti-FRα IgE antibody MOv18 (Rudman, Josephs et al. 2011).

Following the experiments outlined by Rudman et al., who evaluated the potential of the FRα-specific monoclonal antibody MOv18 to mediate type 1 hypersensitivity in the blood of patients with ovarian cancer, it would be imperative to evaluate any

IgE crosslinking in the presence of soluble multivalent HMW-MAA shed in human sera or attached to circulating tumor cells or tumor cell fragments in patient blood (Rudman, Josephs et al. 2011). HMW-MAA is a large antigen which has been previously reported to be present in patient blood (Vergilis, Szarek et al. 2005), and if it circulates in a multivalent form crosslinking may be a special concern because of lack of precise information on the 225.28S antibody epitope (Wilson, Imai et al. 1981; Kantor, Albino et al. 1986; Mittelman, Tiwari et al. 2004), which could be present on the circulating form of the antigen and/or possibly be a repeating determinant. These safety studies would include monitoring early signs of basophil activation from patient blood, along with evaluations of *ex vivo* mast cell degranulation in patient sera together with HMW-MAA IgE to assess the overall safety of this molecule in patients with melanoma. These safety studies, along with those evaluating efficacy, toxicity, pharmacokinetics, and bio-distribution, combined with the evaluations presented in this thesis, would permit the assessments of the potential utility of antibodies targeting HMW-MAA as therapeutic agents.

6.6 Concluding Remarks

The humoral immune response plays a vital role in maintaining health, particularly with respect to bacterial and parasitic infections. The prevalence, role and potency of humoral immune responses are not well understood in relation to malignancy. The work herein has shown that there is an antibody response to melanoma present in patient memory B cells. Examination of the reactivity of antibodies derived from patient memory B cells to melanoma cell lines that originated from different individuals, rather than from autologous tumor cells, using a novel cell-based ELISA revealed that these responses are present among the patient cohort examined, although are decreased with increased disease staging. While the potency of such antibody responses in patients to mount an effective anti-tumor response is unclear, one patient-derived monoclonal antibody was characterized and found to bind to melanoma cells but not to dermal fibroblasts and melanocytes, and was able to mediate cytotoxicity against melanoma cells *in vitro*. This work displays the potential of patient-derived melanoma antibodies to have cytotoxic effects against tumor cells, and points to this previously unexplored arm of adaptive immunity as a potential source of antibodies to be evaluated for passive immunotherapy, or as a source of anti-tumor immunity to be activated against melanoma through future novel therapeutic strategies.

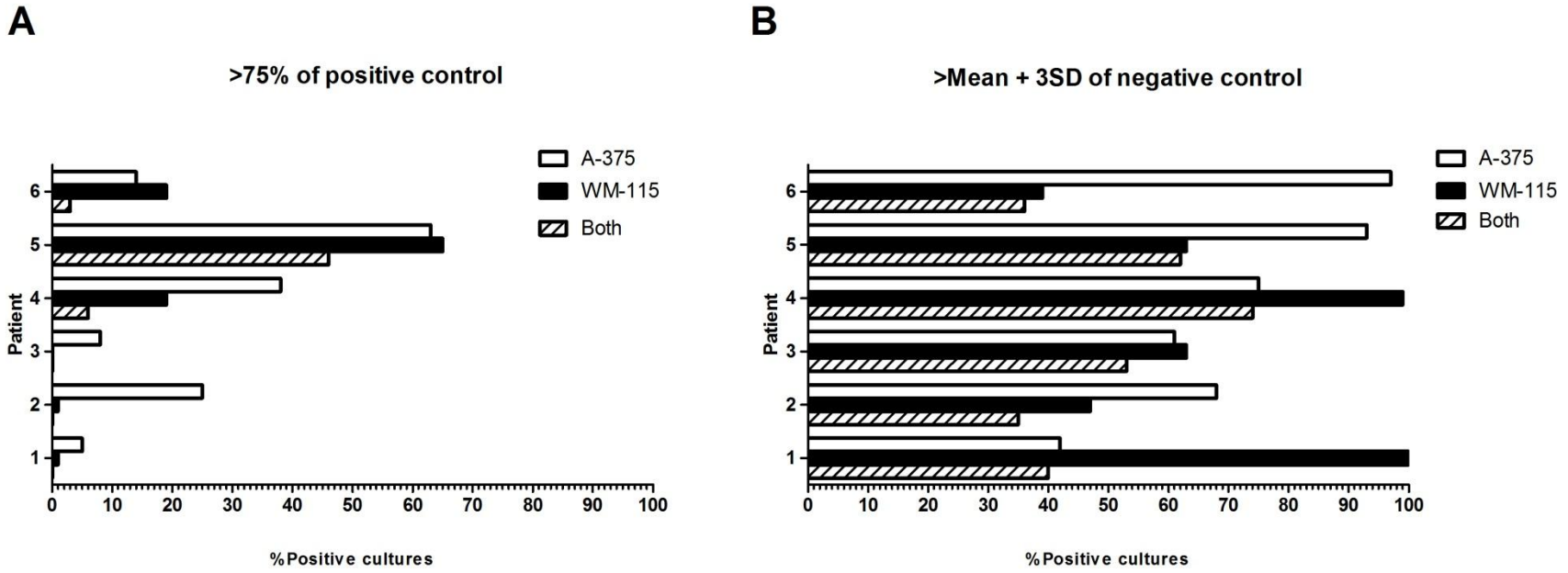
Additionally, evaluations of engineered IgE antibodies targeting the tumor associated antigen HMW-MAA may provide another strategy to harness the immune system against cancer by exploiting the responses normally mediated by antibodies of the IgE class against innocuous allergens and parasites. The exploration of strategies to harness patient immune responses merits further attention in the design of effective immunotherapies, whether they are focused on

activating natural humoral immune responses in the host, using the antibody repertoire of patients as a means to discovering novel monoclonal antibodies, or exploiting the effector functions of a class of antibodies, other than the conventionally used IgG, against solid tumors.

Finally, this thesis has presented a highly translational approach to the discovery and development of monoclonal antibodies for the treatment of cancer through the study of patient specimens and human immune cells. These approaches have included the characterization of humoral immunity in melanoma patients which led to pathways to identify novel tumor-specific antibodies with potential cytotoxic activity against tumor cells, and also the evaluation of antibodies of the IgG and IgE classes targeting a melanoma antigen to recruit human immune cells to mediate ADCP and ADCC against tumor cells. All of these findings are aimed at characterizing and harnessing the host immune response against cancer with the hope of contributing knowledge that translates into new directions in cancer treatment that may hold future therapeutic value for patients.

Appendix A

Comparison of criteria used to define melanoma-reactive antibodies for 6 patients. The criteria used in this thesis to define melanoma-reactive antibodies were compared to the criteria previously used by others when evaluating antibody reactivity by ELISA (Punt, Barbuto et al. 1994; Zhang, Lake et al. 1995; Zhang, Casiano et al. 2003). Melanoma-reactive antibody cultures were defined in this thesis as those cultures with absorbance values greater than that of 75% of the positive control antibody. The percentage of antibody cultures with melanoma-reactive antibodies (positive cultures) was calculated for 6 patients (n=60 culture wells per patient) using these criteria (A). Results for the same 6 patients were also analyzed using the criteria used by others, i.e. mean absorbance value of the non-specific control antibody (B).



Appendix B

Alignment of the patient-derived antibody clone M80F2 variable regions to germline human immunoglobulin. Sequence alignment and identification mutation sites were performed for variable heavy chains (A) and light chains (B) using the IMGT/V-QUEST software (Brochet, Lefranc et al. 2008).

A

M80F2 V_H

Sequence compared with the human IG set from the IMGT reference directory

```
>M80F2H
gaggtgcagctggtggagtctgggggaggcctggtacagcctgggggtccctgagactc
tcctgtgcagcctctggattcacctttaggcactatgccatcagttgggtccgccaggct
ccagggaaggggctggagtgggtctcaggtctgagtggtagtggaaataggacatactac
gcagactccgtgaagggccggttcaccatctccagagacaattccgagaacacgctgttt
ttgcaaatgaacagcctgagagccgaggacacggccgtgtattactgtgcgaaagatcgc
cgagtgggagctaccttcgtctttgactcctggggccagggaaccctggtcaccgtctcc
ccag
```

Result summary:	**Productive IGH rearranged sequence** (no stop codon and in-frame junction)		
V-GENE and allele	Homsap IGHV3-23*04 F	score = 1282	identity = **94,10%** (271/288 nt)
J-GENE and allele	Homsap IGHJ4*02 F (a)	score = 195	identity = 89,58% (43/48 nt)
D-GENE and allele by IMGT/JunctionAnalysis	Homsap IGHD1-26*01 F	D-REGION is in reading frame 1	
FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION	[25.17.38.11]	[8.8.14]	CAKDRRVGATFVFDSW

(a) Other possibilities: Homsap_IGHJ5*02 (highest number of consecutive identical nucleotides)

---

1. Alignment for V-GENE and allele identification

Closest V-REGIONs (evaluated from the V-REGION first nucleotide to the 2nd-CYS codon)

	Score	Identity
AJ879486 Homsap IGHV3-23*04 F	1282	94,10% (271/288 nt)
M99660 Homsap IGHV3-23*01 F	1273	93,75% (270/288 nt)
M35415 Homsap IGHV3-23*02 F	1255	93,06% (268/288 nt)
AY757302 Homsap IGHV3-23*05 F	1237	92,36% (266/288 nt)
U29481 Homsap IGHV3-23*03 F	1219	91,67% (264/288 nt)

Alignment with FR-IMGT and CDR-IMGT delimitations

```
                                  <---------------------------------- FR1-
IMGT ---------------
M80F2H
gaggtgcagctggtggagtctgggggga...ggcctggtacagcctgggggtccctgaga
AJ879486 Homsap IGHV3-23*04 F       --------------------------...---t--------
-----------------
```

```
M99660 Homsap IGHV3-23*01 F         ------------t--------------...---t--------
------------------
M35415 Homsap IGHV3-23*02 F         ------------t--------------...---t--------
------------------
AY757302 Homsap IGHV3-23*05 F       ------------t--------------...---t--------
------------------
U29481 Homsap IGHV3-23*03 F         ------------t--------------...---t--------
------------------

                                    -----------------> CDR1-IMGT
___________<-----
M80F2H
ctctcctgtgcagcctctggattcaccttt.............aggcactatgccatcagt
AJ879486 Homsap IGHV3-23*04 F       ------------------------------
............--cag---------g--c
M99660 Homsap IGHV3-23*01 F         ------------------------------
............--cag---------g--c
M35415 Homsap IGHV3-23*02 F         ------------------------------
............--cag---------g--c
AY757302 Homsap IGHV3-23*05 F       ------------------------------
............--cag---------g--c
U29481 Homsap IGHV3-23*03 F         ------------------------------
............--cag---------g--c

                                    --------------- FR2-IMGT -----------------
-->___________ CDR
M80F2H
tgggtccgccaggctccagggaaggggctggagtgggtctcaggtctgagtggtagt...
AJ879486 Homsap IGHV3-23*04 F       ----------------------------------------
-c-a-t---------...
M99660 Homsap IGHV3-23*01 F         ----------------------------------------
-c-a-t---------...
M35415 Homsap IGHV3-23*02 F         ----------------------------------------
-c-a-t---------...
AY757302 Homsap IGHV3-23*05 F       ----------------------------------------
-c-a-tta-a-c---...
U29481 Homsap IGHV3-23*03 F         ----------------------------------------
-t-a-tta-a-cg--...

                                    2-IMGT ________<--------------------------
------------------
M80F2H
...ggaaataggacatactacgcagactccgtgaag...ggccggttcaccatctccaga
AJ879486 Homsap IGHV3-23*04 F       ...--tgg---c------------------------...---
------------------
M99660 Homsap IGHV3-23*01 F         ...--tgg---c------------------------...---
------------------
M35415 Homsap IGHV3-23*02 F         ...--tgg---c----------g-------------...---
--------------a---
AY757302 Homsap IGHV3-23*05 F       ...--t-g---c--------t---------------...---
------------------
U29481 Homsap IGHV3-23*03 F         ...--t-g---c--------t---------------...---
------------------

                                    --------- FR3-IMGT ----------------------
------------------
M80F2H
gacaattccgagaacacgctgtttttgcaaatgaacagcctgagagccgaggacacggcc
AJ879486 Homsap IGHV3-23*04 F       ---------a------------a-c-----------------
------------------
M99660 Homsap IGHV3-23*01 F         ---------a------------a-c-----------------
------------------
M35415 Homsap IGHV3-23*02 F         ---------a------------a-c-----------------
------------------
AY757302 Homsap IGHV3-23*05 F       ---------a------------a-c-----------------
------------------
U29481 Homsap IGHV3-23*03 F         --t------a------------a-c-----------------
------------------
```

```
                                    ---------->_________________ CDR3-IMGT
_______________
M80F2H
gtgtattactgtgcgaaagatcgccgagtgggagctaccttcgtctttgactcctggggc
AJ879486 Homsap IGHV3-23*04 F       --a-----------------
M99660 Homsap IGHV3-23*01 F         --a-----------------
M35415 Homsap IGHV3-23*02 F         --a-----------------
AY757302 Homsap IGHV3-23*05 F       --a---------------
U29481 Homsap IGHV3-23*03 F         --a---------------


M80F2H                              cagggaaccctggtcaccgtctccccag
AJ879486 Homsap IGHV3-23*04 F
M99660 Homsap IGHV3-23*01 F
M35415 Homsap IGHV3-23*02 F
AY757302 Homsap IGHV3-23*05 F
U29481 Homsap IGHV3-23*03 F
```

---

3. Alignment for J-GENE and allele identification

Closest J-REGIONs

	Score	Identity
X86355 Homsap IGHJ4*02 F	195	89,58% (43/48 nt)
J00256 Homsap IGHJ4*01 F	186	87,50% (42/48 nt)
M25625 Homsap IGHJ4*03 F	177	85,42% (41/48 nt)
J00256 Homsap IGHJ5*01 F	174	82,35% (42/51 nt)
X86355 Homsap IGHJ5*02 F	174	82,35% (42/51 nt)

Note that the highest number of consecutive identical nucleotides has been found in the alignment with Homsap_IGHJ5*02

Alignment

```
M80F2H
tcgccgagtgggagctaccttcgtctttgactcctggggccagggaaccctggtcaccgt
X86355 Homsap IGHJ4*02 F            .....................a-ta--------a---------
------------------
J00256 Homsap IGHJ4*01 F            .....................a-ta--------a---------
a-----------------
M25625 Homsap IGHJ4*03 F            .....................g-ta--------a---------
a--g--------------
J00256 Homsap IGHJ5*01 F            .................a-aa-tgg--c--------------
a-----------------
X86355 Homsap IGHJ5*02 F            .................a-aa-tgg--c---c----------
------------------

M80F2H                              ctccccag
X86355 Homsap IGHJ4*02 F            ----t---
J00256 Homsap IGHJ4*01 F            ----t---
M25625 Homsap IGHJ4*03 F            ----t---
J00256 Homsap IGHJ5*01 F            ----t---
X86355 Homsap IGHJ5*02 F            ----t---
```

---

4. Results of IMGT/JunctionAnalysis

Maximum number of accepted mutations in: 3'V-REGION = 2, D-REGION = 4, 5'J-REGION = 2
Maximum number of accepted D-GENE = 1

Analysis of the JUNCTION

D-REGION is in reading frame 1.

Click on mutated (underlined) nucleotide to see the original one:

Input	V name	3'V-REGION	P	N1	D-REGION	N2	5'J-REGION	J name	D name	V mut	D mut	J mut	Ngc
M80F2H	Homsap IGHV3-23*04	tgtgcgaaaga	tc	gccg	.....agtggagctac...	ctctcgt	....ctttgactcctgg	Homsap IGHJ4*02	Homsap IGHD1-26*01	0	0	1	7/10

Translation of the JUNCTION

Click on mutated (underlined) amino acid to see the original one:

	104	105	106	107	108	109	110	111	112.1	112	113	114	115	116	117	118	Frame	CDR3-IMGT length	Molecular mass	pI
	C	A	K	D	R	R	V	G	A	T	F	V	F	D	S	W				
M80F2H	tgt	gcg	aaa	gat	cgc	cga	gtg	gga	gct	acc	ttc	gtc	ttt	gac	tcc	tgg	+	14	1,858.11	8.23

Be aware that some allele reference sequences may be incomplete or from cDNAs. In those cases, IMGT/JunctionAnalysis uses automatically the allele *01 for the analysis of the JUNCTION.

5. Sequence of the JUNCTION ('nt' and 'AA')

```
104   105   106   107   108   109   110   111   112.1 112   113   114   115
116   117   118
 C     A     K     D     R     R     V     G     A     T     F     V     F
D     S     W
tgt   gcg   aaa   gat   cgc   cga   gtg   gga   gct   acc   ttc   gtc   ttt
gac   tcc   tgg
```

Input for IMGT/JunctionAnalysis

```
>M80F2H,Homsap_IGHV3-23*04,Homsap_IGHJ4*02
tgtgcgaaagatcgccgagtgggagctaccttcgtctttgactcctgg
```

---

6. V-REGION alignment according to the IMGT unique numbering

```
                                  <----------------------------------------
----  FR1 - IMGT
                                  1               5                   10
15
M80F2H                            gag gtg cag ctg gtg gag tct ggg gga ...
ggc ctg gta cag cct
AJ879486 Homsap IGHV3-23*04 F     --- --- --- --- --- --- --- --- --- ... --
- t-- --- --- ---
M99660 Homsap IGHV3-23*01 F       --- --- --- --- t-- --- --- --- --- ... --
- t-- --- --- ---
M35415 Homsap IGHV3-23*02 F       --- --- --- --- t-- --- --- --- --- ... --
- t-- --- --- ---
AY757302 Homsap IGHV3-23*05 F     --- --- --- --- t-- --- --- --- --- ... --
- t-- --- --- ---
U29481 Homsap IGHV3-23*03 F       --- --- --- --- t-- --- --- --- --- ... --
- t-- --- --- ---


                                  ----------------------------------------
> _______________
                                                  20                  25
30
M80F2H                            ggg ggg tcc ctg aga ctc tcc tgt gca gcc
tct gga ttc acc ttt
AJ879486 Homsap IGHV3-23*04 F     --- --- --- --- --- --- --- --- --- --- --
- --- --- --- ---
M99660 Homsap IGHV3-23*01 F       --- --- --- --- --- --- --- --- --- --- --
- --- --- --- ---
M35415 Homsap IGHV3-23*02 F       --- --- --- --- --- --- --- --- --- --- --
- --- --- --- ---
AY757302 Homsap IGHV3-23*05 F     --- --- --- --- --- --- --- --- --- --- --
- --- --- --- ---
U29481 Homsap IGHV3-23*03 F       --- --- --- --- --- --- --- --- --- --- --
- --- --- --- ---


                                  __  CDR1 - IMGT  _____________ <---------
-----------------
                                                  35                  40
45
M80F2H                            ... ... ... ... agg cac tat gcc atc agt
tgg gtc cgc cag gct
```

```
AJ879486 Homsap IGHV3-23*04 F       ... ... ... ... --c ag- --- --- --g --c --
- --- --- --- ---
M99660 Homsap IGHV3-23*01 F         ... ... ... ... --c ag- --- --- --g --c --
- --- --- --- ---
M35415 Homsap IGHV3-23*02 F         ... ... ... ... --c ag- --- --- --g --c --
- --- --- --- ---
AY757302 Homsap IGHV3-23*05 F       ... ... ... ... --c ag- --- --- --g --c --
- --- --- --- ---
U29481 Homsap IGHV3-23*03 F         ... ... ... ... --c ag- --- --- --g --c --
- --- --- --- ---


                                     FR2 - IMGT  ------------------------>
_____________  CDR2
                                                 50                  55
60
M80F2H                              cca ggg aag ggg ctg gag tgg gtc tca ggt
ctg agt ggt agt ...
AJ879486 Homsap IGHV3-23*04 F       --- --- --- --- --- --- --- --- --- -c- a-
t --- --- --- ...
M99660 Homsap IGHV3-23*01 F         --- --- --- --- --- --- --- --- --- -c- a-
t --- --- --- ...
M35415 Homsap IGHV3-23*02 F         --- --- --- --- --- --- --- --- --- -c- a-
t --- --- --- ...
AY757302 Homsap IGHV3-23*05 F       --- --- --- --- --- --- --- --- --- -c- a-
t ta- a-c --- ...
U29481 Homsap IGHV3-23*03 F         --- --- --- --- --- --- --- --- --- -t- a-
t ta- a-c g-- ...


                                     - IMGT  __________ <----------------------
------------------
                                                 65                  70
75
M80F2H                              ... gga aat agg aca tac tac gca gac tcc
gtg aag ... ggc cgg
AJ879486 Homsap IGHV3-23*04 F       ... --t gg- --c --- --- --- --- --- --- --
- --- ... --- ---
M99660 Homsap IGHV3-23*01 F         ... --t gg- --c --- --- --- --- --- --- --
- --- ... --- ---
M35415 Homsap IGHV3-23*02 F         ... --t gg- --c --- --- --- -g- --- --- --
- --- ... --- ---
AY757302 Homsap IGHV3-23*05 F       ... --t -g- --c --- --- --t --- --- --- --
- --- ... --- ---
U29481 Homsap IGHV3-23*03 F         ... --t -g- --c --- --- --t --- --- --- --
- --- ... --- ---


                                    ------------------------------  FR3 -
IMGT  ---------------
                                                 80                  85
90
M80F2H                              ttc acc atc tcc aga gac aat tcc gag aac
acg ctg ttt ttg caa
AJ879486 Homsap IGHV3-23*04 F       --- --- --- --- --- --- --- --- a-- --- --
- --- -a- c-- ---
M99660 Homsap IGHV3-23*01 F         --- --- --- --- --- --- --- --- a-- --- --
- --- -a- c-- ---
M35415 Homsap IGHV3-23*02 F         --- --- --- --a --- --- --- --- a-- --- --
- --- -a- c-- ---
AY757302 Homsap IGHV3-23*05 F       --- --- --- --- --- --- --- --- a-- --- --
- --- -a- c-- ---
U29481 Homsap IGHV3-23*03 F         --- --- --- --- --- --t --- --- a-- --- --
- --- -a- c-- ---


                                    ----------------------------------------
------------> ____
                                                 95                  100
104
M80F2H                              atg aac agc ctg aga gcc gag gac acg gcc
gtg tat tac tgt gcg
AJ879486 Homsap IGHV3-23*04 F       --- --- --- --- --- --- --- --- --- --- --
a --- --- --- ---
```

```
M99660 Homsap IGHV3-23*01 F          --- --- --- --- --- --- --- --- --- --- --
a --- --- --- ---
M35415 Homsap IGHV3-23*02 F          --- --- --- --- --- --- --- --- --- --- --
a --- --- --- ---
AY757302 Homsap IGHV3-23*05 F        --- --- --- --- --- --- --- --- --- --- --
a --- --- --- ---
U29481 Homsap IGHV3-23*03 F          --- --- --- --- --- --- --- --- --- --- --
a --- --- --- ---


                                                 _________________  CDR3 - IMGT
_________________

M80F2H                               aaa gat cgc cga gtg gga gct acc ttc gtc
ttt gac tcc tgg ggc
AJ879486 Homsap IGHV3-23*04 F        --- --
M99660 Homsap IGHV3-23*01 F          --- --
M35415 Homsap IGHV3-23*02 F          --- --
AY757302 Homsap IGHV3-23*05 F        ---
U29481 Homsap IGHV3-23*03 F          ---


M80F2H                               cag gga acc ctg gtc acc gtc tcc cca g
AJ879486 Homsap IGHV3-23*04 F
M99660 Homsap IGHV3-23*01 F
M35415 Homsap IGHV3-23*02 F
AY757302 Homsap IGHV3-23*05 F
U29481 Homsap IGHV3-23*03 F
```

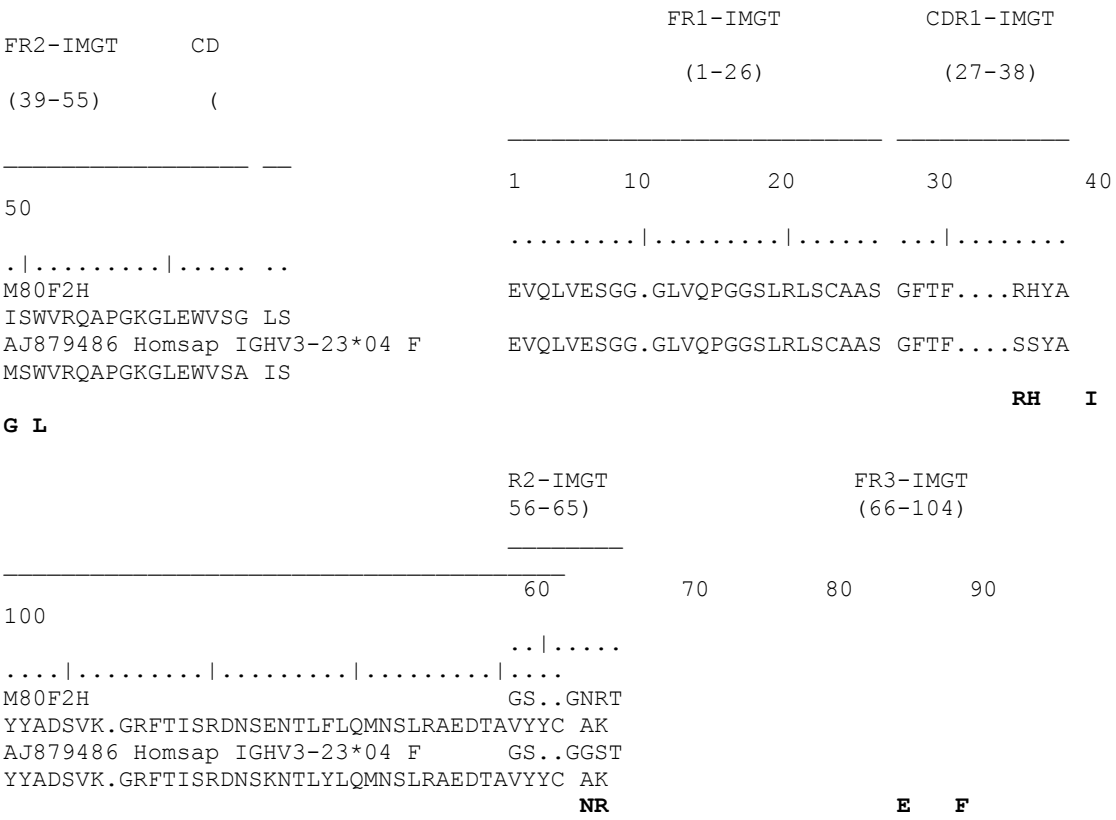
7. V-REGION translation

```
                                     <-----------------------------------------
----  FR1 - IMGT
                                     1               5                   10
15
                                      E   V   Q   L   V   E   S   G   G       G
L   V   Q   P
M80F2H                               gag gtg cag ctg gtg gag tct ggg gga ...
ggc ctg gta cag cct

AJ879486 Homsap IGHV3-23*04 F        --- --- --- --- --- --- --- --- --- ... --
- t-- --- --- ---


                                     ----------------------------------------
> _________________
                                                   20                  25
30
                                      G   G   S   L   R   L   S   C   A   A   S
G   F   T   F
M80F2H                               ggg ggg tcc ctg aga ctc tcc tgt gca gcc
tct gga ttc acc ttt

AJ879486 Homsap IGHV3-23*04 F        --- --- --- --- --- --- --- --- --- --- --
- --- --- --- ---


                                     __  CDR1 - IMGT  _______________ <---------
-----------------
                                                   35                  40
45
                                                    R   H   Y   A   I   S   W
V   R   Q   A
M80F2H                               ... ... ... ... agg cac tat gcc atc agt
tgg gtc cgc cag gct
                                                    S   S           M
```

```
AJ879486 Homsap IGHV3-23*04 F
- --- --- --- ---


_____  CDR2

60

S   G   S
M80F2H
ctg agt ggt agt ...

AJ879486 Homsap IGHV3-23*04 F
t --- --- --- ...


------------------

75

K       G   R
M80F2H
gtg aag ... ggc cgg

AJ879486 Homsap IGHV3-23*04 F
- --- ... --- ---


IMGT  ---------------

90

L   F   L   Q
M80F2H
acg ctg ttt ttg caa

Y
AJ879486 Homsap IGHV3-23*04 F
- --- -a- c-- ---


------------> ____

104

Y   Y   C   A
M80F2H
gtg tat tac tgt gcg

AJ879486 Homsap IGHV3-23*04 F
a --- --- --- ---


____________________


D   S   W   G
M80F2H
ttt gac tcc tgg ggc

AJ879486 Homsap IGHV3-23*04 F



M80F2H

AJ879486 Homsap IGHV3-23*04 F
```

```
... ... ... ... --c ag- --- --- --g --c --

 FR2 - IMGT  ------------------------>

                50                  55

 P   G   K   G   L   E   W   V   S   G   L

cca ggg aag ggg ctg gag tgg gtc tca ggt

                                        A   I
--- --- --- --- --- --- --- --- --- -c- a-

 - IMGT  __________ <---------------------

                65                  70

     G   N   R   T   Y   Y   A   D   S   V

... gga aat agg aca tac tac gca gac tcc

        G   S
... --t gg- --c --- --- --- --- --- --- --

-------------------------------  FR3 -

                80                  85

 F   T   I   S   R   D   N   S   E   N   T

ttc acc atc tcc aga gac aat tcc gag aac

                                    K
--- --- --- --- --- --- --- --- a-- --- --

-----------------------------------------

                95                  100

 M   N   S   L   R   A   E   D   T   A   V

atg aac agc ctg aga gcc gag gac acg gcc

--- --- --- --- --- --- --- --- --- --- --

_____________________  CDR3 - IMGT

 K   D   R   R   V   G   A   T   F   V   F

aaa gat cgc cga gtg gga gct acc ttc gtc

--- --


 Q   G   T   L   V   T   V   S   P
cag gga acc ctg gtc acc gtc tcc cca g
```

8. V-REGION protein display



9. V-REGION mutation and AA change table

FR1-IMGT	CDR1-IMGT	FR2-IMGT	CDR2-IMGT	FR3-IMGT	CDR3-IMGT
t34>c	c105>g, S35>R (- - -) a106>c, S36>H (+ - -) g107>a, S36>H (+ - -)	g117>c, M39>I (+ + -) c120>t c164>g, A55>G (- + -)	a166>c, I56>L (+ + +) t168>g, I56>L (+ + +) t186>a g187>a, G63>N (- - -) g188>a, G63>N (- - -) c192>g, S64>R (- - -)	a250>g, K84>E (+ - -) a263>t, Y88>F (- + -) c265>t a303>g	

10. V-REGION mutation and AA change statistics

Nucleotide (nt) mutations

IMGT labels		V-REGION	FR1-IMGT	CDR1-IMGT	FR2-IMGT	CDR2-IMGT	FR3-IMGT	CDR3-IMGT
Nb of positions including IMGT gaps (nt)		**320**	78	36	51	30	117	8
Nb of nucleotides		**296**	75	24	51	24	114	8
Nb of identical nucleotides		**279**	74	21	48	18	110	8
Nb of mutations		**17**	1	3	3	6	4	0
Mutations	**Silent**	**5**	1	0	1	1	2	0
	Nonsilent	**12**	0	3	2	5	2	0
Transitions	**a>g**	**2**	0	0	0	0	2	0
	g>a	**3**	0	1	0	2	0	0
	c>t	**2**	0	0	1	0	1	0
	t>c	**1**	1	0	0	0	0	0
Transversions	**a>c**	**2**	0	1	0	1	0	0
	c>a	**0**	0	0	0	0	0	0
	a>t	**1**	0	0	0	0	1	0
	t>a	**1**	0	0	0	1	0	0
	g>c	**1**	0	0	1	0	0	0
	c>g	**3**	0	1	1	1	0	0
	g>t	**0**	0	0	0	0	0	0
	t>g	**1**	0	0	0	1	0	0

Amino acid (AA) changes

IMGT labels			V-REGION	FR1-IMGT	CDR1-IMGT	FR2-IMGT	CDR2-IMGT	FR3-IMGT	CDR3-IMGT
Nb of positions including IMGT gaps (AA)			**106**	26	12	17	10	39	2
Nb of AA			**98**	25	8	17	8	38	2
Nb of identical AA			**89**	25	6	15	5	36	2
Nb of AA changes			**9**	0	2	2	3	2	0
AA changes	**Very similar**	**(+ + +)**	**1**	0	0	0	1	0	0
	Similar	**(+ + -)**	**1**	0	0	1	0	0	0

		(+ - +)	**0**	0	0	0	0	0	0
	Dissimilar	(+ - -)	**2**	0	1	0	0	1	0
		(- + -)	**2**	0	0	1	0	1	0
		(- - +)	**0**	0	0	0	0	0	0
	Very dissimilar	(- - -)	**3**	0	1	0	2	0	0

B

M80F2 V_L

Sequence compared with the human IG set from the IMGT reference directory

```
>M80F2K
atgacccagactccatccaccctgtctgcatctgtgggagacagagtcaccatcacttgc
cgggcaagtcagaacattaccacctatttcaattggtatcagcaaaaaccagggaaagcc
cctaaactcctgatctatgctgcatccagtttgcaaagtggggtcccatcaaggttcagt
ggcagtggatctgggacagatttcactctcaccatcaccagtctgcaacctgaagatttt
gcaacttactactgtcaacagaggggacgttcggccaagggaccaaggtggaaatcaaa
cga
```

Result summary:	**Productive IGK rearranged sequence** (no stop codon and in-frame junction)		
V-GENE and allele	Homsap IGKV1-39*01 F, or Homsap IGKV1D-39*01 F	score = 1210	identity = **94,07%** (254/270 nt)
J-GENE and allele	Homsap IGKJ1*01 F	score = 181	identity = 97,37% (37/38 nt)
FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION	[23.17.36.10]	[6.3.5]	CQQRGTF

1. Alignment for V-GENE and allele identification

Closest V-REGIONs (evaluated from the V-REGION first nucleotide to the 2nd-CYS codon plus 15 nt of the CDR3-IMGT)

		Score	Identity
X59315	Homsap IGKV1-39*01 F	1210	94,07% (254/270 nt)
X59312	Homsap IGKV1D-39*01 F	1210	94,07% (254/270 nt)
X59318	Homsap IGKV1-39*02 P	1147	91,48% (247/270 nt)
Z00013	Homsap IGKV1-9*01 F	1066	88,15% (238/270 nt)
V01577	Homsap IGKV1-12*01 F	1057	87,78% (237/270 nt)

Alignment with FR-IMGT and CDR-IMGT delimitations

```
                                    <---------------------------------- FR1-
IMGT ---------------
M80F2K
.........atgacccagactccatccaccctgtctgcatctgtgggagacagagtcacc
X59315 Homsap IGKV1-39*01 F         gacatccag---------t--------t--------------
--a---------------
X59312 Homsap IGKV1D-39*01 F        gacatccag---------t--------t--------------
--a---------------
X59318 Homsap IGKV1-39*02 P         gacatccag---------t--------tt-------------
--a---------------
Z00013 Homsap IGKV1-9*01 F          gacatccagt--------t--------tt-------------
--a---------------
V01577 Homsap IGKV1-12*01 F         gacatccag---------t-------tt--g-----------
--a---------------

                                    -----------------> CDR1-IMGT
___________<-----
M80F2K
atcacttgccgggcaagtcagaacatt..................accacctatttcaat
X59315 Homsap IGKV1-39*01 F         ----------------------g----
..................-g--g------a---
X59312 Homsap IGKV1D-39*01 F        ----------------------g----
..................-g--g------a---
X59318 Homsap IGKV1-39*02 P         ----------------------g----
..................-g--g------a---
Z00013 Homsap IGKV1-9*01 F          --------------c------gg----
...................-g--gt-----agcc
V01577 Homsap IGKV1-12*01 F         --------t-----g------ggt---
...................-g--g--gg--agcc

                                    --------------- FR2-IMGT -----------------
-->___________ CDR
M80F2K
tggtatcagcaaaaaccagggaaagcccctaaactcctgatctatgctgca.........
X59315 Homsap IGKV1-39*01 F         -----------g--------------------g---------
---------.........
X59312 Homsap IGKV1D-39*01 F        -----------g--------------------g---------
---------.........
X59318 Homsap IGKV1-39*02 P         -----------g--------------------g---------
---------.........
Z00013 Homsap IGKV1-9*01 F          ----------------------------------g---------
---------.........
V01577 Homsap IGKV1-12*01 F         -----------g--------------------g---------
---------.........

                                    2-IMGT ________<--------------------------
------------------
M80F2K
............tccagtttgcaaagtggggtccca...tcaaggttcagtggcagtgga
X59315 Homsap IGKV1-39*01 F         ............------------------------...---
------------------
X59312 Homsap IGKV1D-39*01 F        ............------------------------...---
------------------
X59318 Homsap IGKV1-39*02 P         ............------------------------...---
------------------
Z00013 Homsap IGKV1-9*01 F          ............----c-------------------...---
--------c---------
V01577 Homsap IGKV1-12*01 F         ............------------------------...---
--------c---------

                                    --------- FR3-IMGT ----------------------
------------------
M80F2K
......tctgggacagatttcactctcaccatcaccagtctgcaacctgaagattttgca
X59315 Homsap IGKV1-39*01 F         ......----------------------------g-------
------------------
X59312 Homsap IGKV1D-39*01 F        ......----------------------------g-------
------------------
X59318 Homsap IGKV1-39*02 P         ......----------------------------g-------
------------------
```

```
Z00013 Homsap IGKV1-9*01 F          ......----------a----------a----g---c---
--g---------------
V01577 Homsap IGKV1-12*01 F         ......----------------------------g---c---
--g---------------

                                    ----------->___ CDR3-IMGT _
M80F2K
acttactactgtcaacagaggggacgttcggccaagggaccaaggtggaaatcaaacga
X59315 Homsap IGKV1-39*01 F         --------------------ttac-gtac-cct-c
X59312 Homsap IGKV1D-39*01 F        --------------------ttac-gtac-cct-c
X59318 Homsap IGKV1-39*02 P         -----t--------gtgtg-ttac-gtacacct-c
Z00013 Homsap IGKV1-9*01 F          -----t------------cttaat-gt-a-cct-c
V01577 Homsap IGKV1-12*01 F         --------t---------gctaac-gt---cct-c
```

3. Alignment for J-GENE and allele identification

Closest J-REGIONs

	Score	Identity
J00242 Homsap IGKJ1*01 F	181	97,37% (37/38 nt)
AF103571 Homsap IGKJ4*02 F	136	84,21% (32/38 nt)
Z70260 Homsap IGKJ2*02 F	127	81,58% (31/38 nt)
J00242 Homsap IGKJ4*01 F	127	81,58% (31/38 nt)
J00242 Homsap IGKJ2*01 F	118	78,95% (30/38 nt)

Alignment

```
M80F2K                              ggggacgttcggccaagggaccaaggtggaaatcaaacga
J00242 Homsap IGKJ1*01 F            -t-------------------------------------
AF103571 Homsap IGKJ4*02 F          -ctc---------gg---------------g-------
Z70260 Homsap IGKJ2*02 F            -t-c--t--t-----g---------c----g-------
J00242 Homsap IGKJ4*01 F            -ctc--t------gg---------------g-------
J00242 Homsap IGKJ2*01 F            -tac--t--t-----g---------c----g-------
```

4. Results of IMGT/JunctionAnalysis

Maximum number of accepted mutations in: 3'V-REGION = 7, 5'J-REGION = 7
Maximum number of accepted D-GENE = 0

Analysis of the JUNCTION

Click on mutated (underlined) nucleotide to see the original one:

Input	V name	3'V-REGION	N	5'J-REGION	J name	Vmut	Jmut	Ngc
M80F2K	Homsap IGKV1-...	tgtcaacagag.....	gg	..ggacgttc	Homsap IGKJ1*01	0	0	2/2

39*01

Translation of the JUNCTION

Click on mutated (underlined) amino acid to see the original one:

	104	105	106	107	116	117	118	Frame	CDR3-IMGT length	Molecular mass	pI
	C	Q	Q	R	G	T	F				
M80F2K	tgt	caa	cag	agg	ggg	acg	ttc	+	5	838.94	8.25

Be aware that some allele reference sequences may be incomplete or from cDNAs. In those cases, IMGT/JunctionAnalysis uses automatically the allele *01 for the analysis of the JUNCTION.

5. Sequence of the JUNCTION ('nt' and 'AA')

```
104 105 106 107 116 117 118
 C   Q   Q   R   G   T   F
tgt caa cag agg ggg acg ttc
```

Input for IMGT/JunctionAnalysis

```
>M80F2K,Homsap_IGKV1-39*01,Homsap_IGKJ1*01
tgtcaacagaggggacgttc
```

6. V-REGION alignment according to the IMGT unique numbering

```
                                    <----------------------------------------
----  FR1 - IMGT
                                    1               5                   10
15
M80F2K                              ... ... ... atg acc cag act cca tcc acc
ctg tct gca tct gtg
X59315 Homsap IGKV1-39*01 F         gac atc cag --- --- --- t-- --- --- t-- --
- --- --- --- --a
X59312 Homsap IGKV1D-39*01 F        gac atc cag --- --- --- t-- --- --- t-- --
- --- --- --- --a
X59318 Homsap IGKV1-39*02 P         gac atc cag --- --- --- t-- --- --- tt- --
- --- --- --- --a
Z00013 Homsap IGKV1-9*01 F          gac atc cag t-- --- --- t-- --- --- tt- --
- --- --- --- --a
V01577 Homsap IGKV1-12*01 F         gac atc cag --- --- --- t-- --- --t t-- g-
- --- --- --- --a
```

```
> _________________                                  ----------------------------------------

30                                                                 20                  25
M80F2K                                               gga gac aga gtc acc atc act tgc cgg gca
agt cag aac att ...
X59315 Homsap IGKV1-39*01 F                          --- --- --- --- --- --- --- --- --- --- --
- --- -g- --- ...
X59312 Homsap IGKV1D-39*01 F                         --- --- --- --- --- --- --- --- --- --- --
- --- -g- --- ...
X59318 Homsap IGKV1-39*02 P                          --- --- --- --- --- --- --- --- --- --- --
- --- -g- --- ...
Z00013 Homsap IGKV1-9*01 F                           --- --- --- --- --- --- --- --- --- --c --
- --- gg- --- ...
V01577 Homsap IGKV1-12*01 F                          --- --- --- --- --- --- --- --t --- --g --
- --- ggt --- ...

                                                     __  CDR1 - IMGT  _______________ <---------
-----------------
                                                                   35                  40
45
M80F2K                                               ... ... ... ... ... acc acc tat ttc aat
tgg tat cag caa aaa
X59315 Homsap IGKV1-39*01 F                          ... ... ... ... ... -g- -g- --- --a --- --
- --- --- --g ---
X59312 Homsap IGKV1D-39*01 F                         ... ... ... ... ... -g- -g- --- --a --- --
- --- --- --g ---
X59318 Homsap IGKV1-39*02 P                          ... ... ... ... ... -g- -g- --- --a --- --
- --- --- --g ---
Z00013 Homsap IGKV1-9*01 F                           ... ... ... ... ... -g- -gt --- --a gcc --
- --- --- --- ---
V01577 Homsap IGKV1-12*01 F                          ... ... ... ... ... -g- -g- -gg --a gcc --
- --- --- --g ---

                                                      FR2 - IMGT  ------------------------->
_________________  CDR2
                                                                   50                  55
60
M80F2K                                               cca ggg aaa gcc cct aaa ctc ctg atc tat
gct gca ... ... ...
X59315 Homsap IGKV1-39*01 F                          --- --- --- --- --- --g --- --- --- --- --
- --- ... ... ...
X59312 Homsap IGKV1D-39*01 F                         --- --- --- --- --- --g --- --- --- --- --
- --- ... ... ...
X59318 Homsap IGKV1-39*02 P                          --- --- --- --- --- --g --- --- --- --- --
- --- ... ... ...
Z00013 Homsap IGKV1-9*01 F                           --- --- --- --- --- --g --- --- --- --- --
- --- ... ... ...
V01577 Homsap IGKV1-12*01 F                          --- --- --- --- --- --g --- --- --- --- --
- --- ... ... ...

                                                      - IMGT  __________ <---------------------
------------------
                                                                   65                  70
75
M80F2K                                               ... ... ... ... tcc agt ttg caa agt ggg
gtc cca ... tca agg
X59315 Homsap IGKV1-39*01 F                          ... ... ... ... --- --- --- --- --- --- --
- --- ... --- ---
X59312 Homsap IGKV1D-39*01 F                         ... ... ... ... --- --- --- --- --- --- --
- --- ... --- ---
X59318 Homsap IGKV1-39*02 P                          ... ... ... ... --- --- --- --- --- --- --
- --- ... --- ---
Z00013 Homsap IGKV1-9*01 F                           ... ... ... ... --- -c- --- --- --- --- --
- --- ... --- ---
V01577 Homsap IGKV1-12*01 F                          ... ... ... ... --- --- --- --- --- --- --
- --- ... --- ---
```

```
IMGT  ---------------                                  ------------------------------  FR3 -

                                                                  80                  85
90
M80F2K                                                 ttc agt ggc agt gga ... ... tct ggg aca
gat ttc act ctc acc
X59315 Homsap IGKV1-39*01 F                            --- --- --- --- --- ... ... --- --- --- --
- --- --- --- ---
X59312 Homsap IGKV1D-39*01 F                           --- --- --- --- --- ... ... --- --- --- --
- --- --- --- ---
X59318 Homsap IGKV1-39*02 P                            --- --- --- --- --- ... ... --- --- --- --
- --- --- --- ---
Z00013 Homsap IGKV1-9*01 F                             --- --c --- --- --- ... ... --- --- --- --
a --- --- --- --a
V01577 Homsap IGKV1-12*01 F                            --- --c --- --- --- ... ... --- --- --- --
- --- --- --- ---


------------> ___                                      ----------------------------------------
                                                                  95                  100
104
M80F2K                                                 atc acc agt ctg caa cct gaa gat ttt gca
act tac tac tgt caa
X59315 Homsap IGKV1-39*01 F                            --- -g- --- --- --- --- --- --- --- --- --
- --- --- --- ---
X59312 Homsap IGKV1D-39*01 F                           --- -g- --- --- --- --- --- --- --- --- --
- --- --- --- ---
X59318 Homsap IGKV1-39*02 P                            --- -g- --- --- --- --- --- --- --- --- --
- --t --- --- --g
Z00013 Homsap IGKV1-9*01 F                             --- -g- --c --- --g --- --- --- --- --- --
- --t --- --- ---
V01577 Homsap IGKV1-12*01 F                            --- -g- --c --- --g --- --- --- --- --- --
- --- --t --- ---


                                                        CDR3 -  IMGT _

M80F2K                                                 cag agg ggg acg ttc ggc caa ggg acc aag
gtg gaa atc aaa cga
X59315 Homsap IGKV1-39*01 F                            --- --t tac -gt ac- cct -c
X59312 Homsap IGKV1D-39*01 F                           --- --t tac -gt ac- cct -c
X59318 Homsap IGKV1-39*02 P                            tgt g-t tac -gt aca cct -c
Z00013 Homsap IGKV1-9*01 F                             --- ctt aat -gt -a- cct -c
V01577 Homsap IGKV1-12*01 F                            --- gct aac -gt --- cct -c
```

---

7. V-REGION translation

```
                                                       <----------------------------------------

----  FR1 - IMGT                                       1               5                   10

15                                                                  M   T   Q   T   P   S   T   L

S   A   S   V                                          ... ... ... atg acc cag act cca tcc acc
M80F2K
ctg tct gca tct gtg                                     D   I   Q               S           S
                                                       gac atc cag --- --- --- t-- --- --- t-- --
X59315 Homsap IGKV1-39*01 F
- --- --- --- --a                                      ----------------------------------------


> _________________                                                 20                  25

30                                                      G   D   R   V   T   I   T   C   R   A   S

Q   N   I
```

```
M80F2K                                  gga gac aga gtc acc atc act tgc cgg gca
agt cag aac att ...

S
X59315 Homsap IGKV1-39*01 F             --- --- --- --- --- --- --- --- --- --- --
- --- -g- --- ...

                                        __  CDR1 - IMGT  _______________ <---------
-----------------
                                                        35                  40
45
                                                             T   T   Y   F   N   W
Y   Q   Q   K
M80F2K                                  ... ... ... ... ... acc acc tat ttc aat
tgg tat cag caa aaa
                                                             S   S       L
X59315 Homsap IGKV1-39*01 F             ... ... ... ... ... -g- -g- --- --a --- --
- --- --- --g ---

                                         FR2 - IMGT  ------------------------>
_____________  CDR2
                                                        50                  55
60
                                         P   G   K   A   P   K   L   L   I   Y   A
A
M80F2K                                  cca ggg aaa gcc cct aaa ctc ctg atc tat
gct gca ... ... ...

X59315 Homsap IGKV1-39*01 F             --- --- --- --- --- --g --- --- --- --- --
- --- ... ... ...

                                         - IMGT  __________ <---------------------
------------------
                                                        65                  70
75
                                                         S   S   L   Q   S   G   V
P       S   R
M80F2K                                  ... ... ... ... tcc agt ttg caa agt ggg
gtc cca ... tca agg

X59315 Homsap IGKV1-39*01 F             ... ... ... ... --- --- --- --- --- --- --
- --- ... --- ---

                                        ------------------------------  FR3 -
IMGT  ---------------
                                                        80                  85
90
                                         F   S   G   S   G           S   G   T   D
F   T   L   T
M80F2K                                  ttc agt ggc agt gga ... ... tct ggg aca
gat ttc act ctc acc

X59315 Homsap IGKV1-39*01 F             --- --- --- --- --- ... ... --- --- --- --
- --- --- --- ---

                                        ----------------------------------------
------------> ___
                                                        95                  100
104
                                         I   T   S   L   Q   P   E   D   F   A   T
Y   Y   C   Q
M80F2K                                  atc acc agt ctg caa cct gaa gat ttt gca
act tac tac tgt caa
                                             S
X59315 Homsap IGKV1-39*01 F             --- -g- --- --- --- --- --- --- --- --- --
- --- --- --- ---

                                         CDR3 -  IMGT _
```

```
                                                Q   R   G   T   F   G   Q   G   T   K   V
E   I   K   R
M80F2K                                          cag agg ggg acg ttc ggc caa ggg acc aag
gtg gaa atc aaa cga
                                                    S   Y   S   T   P
X59315 Homsap IGKV1-39*01 F                     --- --t tac -gt ac- cct -c
```

8. V-REGION protein display

```
                                                        FR1-IMGT            CDR1-IMGT
FR2-IMGT     CD
                                                          (1-26)              (27-38)
(39-55)       (
                                                _____________________________ ___________
_________________ __
                                                1       10        20         30         40
50
                                                .........|.........|...... ...|........
.|.........|..... ..
M80F2K                                          ...MTQTPSTLSASVGDRVTITCRAS QNI......TTY
FNWYQQKPGKAPKLLIY AA
X59315 Homsap IGKV1-39*01 F                    DIQMTQSPSSLSASVGDRVTITCRAS QSI......SSY
LNWYQQKPGKAPKLLIY AA
                                                      T  T                   N       TT  F

                                                R2-IMGT                 FR3-IMGT
                                                56-65)                  (66-104)
                                                ________
_____________________________________
                                                 60        70        80        90
100
                                                ..|.....
....|.........|.........|.........|....
M80F2K                                          .......S
SLQSGVP.SRFSGSG..SGTDFTLTITSLQPEDFATYYC QQ
X59315 Homsap IGKV1-39*01 F                    .......S
SLQSGVP.SRFSGSG..SGTDFTLTISSLQPEDFATYYC QQSYSTP
                                                                                    T
```

9. V-REGION mutation and AA change table

FR1-IMGT	CDR1-IMGT	FR2-IMGT	CDR2-IMGT	FR3-IMGT	CDR3-IMGT
t19>a, S7>T (+ - +) t28>a, S10>T (+ - +) a45>g	g83>a, S28>N (- - -) g107>c, S36>T (+ - +) g110>c, S37>T (+ - +)	a117>c, L39>F (+ - -) g132>a g153>a		g275>c, S92>T (+ - +)	t321>g, S107>R (- - -) t322>g, Y108>G (+ - -) a323>g, Y108>G (+ - -) c324>g, Y108>G (+ -

					-) g326>c, S109>T (+ - +) t327>g, S109>T (+ - +)

10. V-REGION mutation and AA change statistics

Nucleotide (nt) mutations

IMGT labels		V-REGION	FR1-IMGT	CDR1-IMGT	FR2-IMGT	CDR2-IMGT	FR3-IMGT	CDR3-IMGT
Nb of positions including IMGT gaps (nt)		**320** (327)	78	36	51	30	117	8 (15)
Nb of nucleotides		**263** (270)	69	18	51	9	108	8 (15)
Nb of identical nucleotides		**253** (254)	66	15	48	9	107	8 (9)
Nb of mutations		**10** (16)	3	3	3	0	1	0 (6)
Mutations	**Silent**	**3**	1	0	2	0	0	0
	Nonsilent	**7** (13)	2	3	1	0	1	0 (6)
Transitions	**a>g**	**1** (2)	1	0	0	0	0	0 (1)
	g>a	**3**	0	1	2	0	0	0
	c>t	**0**	0	0	0	0	0	0
	t>c	**0**	0	0	0	0	0	0
Transversions	**a>c**	**1**	0	0	1	0	0	0
	c>a	**0**	0	0	0	0	0	0
	a>t	**0**	0	0	0	0	0	0
	t>a	**2**	2	0	0	0	0	0
	g>c	**3** (4)	0	2	0	0	1	0 (1)
	c>g	**0** (1)	0	0	0	0	0	0 (1)
	g>t	**0**	0	0	0	0	0	0
	t>g	**0** (3)	0	0	0	0	0	0 (3)

Amino acid (AA) changes

IMGT labels	V-REGION	FR1-IMGT	CDR1-IMGT	FR2-IMGT	CDR2-IMGT	FR3-IMGT	CDR3-IMGT

Nb of positions including IMGT gaps (AA)			**106** (109)	26	12	17	10	39	2 (5)
Nb of AA			**87** (90)	23	6	17	3	36	2 (5)
Nb of identical AA			**80**	21	3	16	3	35	2
Nb of AA changes			**7** (10)	2	3	1	0	1	0 (3)
AA changes	**Very similar**	**(+ + +)**	**0**	0	0	0	0	0	0
	Similar	**(+ + -)**	**0**	0	0	0	0	0	0
		(+ - +)	**5** (6)	2	2	0	0	1	0 (1)
	Dissimilar	**(+ - -)**	**1** (2)	0	0	1	0	0	0 (1)
		(- + -)	**0**	0	0	0	0	0	0
		(- - +)	**0**	0	0	0	0	0	0
	Very dissimilar	**(- - -)**	**1** (2)	0	1	0	0	0	0 (1)

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